

Protection by neuronal growth factors against hyperglycemia induced changes in rat dorsal root ganglion neurons

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II Abbreviations

AGE:	advanced glycation end products
ALS:	amyotrophic lateral sclerosis
ATF3:	activating transcription factor 3
ARTN:	artemin (neublastin or enovin)
BBB:	blood brain barrier
BDNF:	brain-derived growth factor
BSA:	bovine serum albumin
CNS:	Central nervous system
CRE:	ATF/cyclic AMP response element
CREB:	cAMP response element binding-protein
DAPI:	4', 6-Diamidino-2-phenylindole
DMEM:	Dulbecco's Modified Eagle Medium
DRG:	dorsal root ganglion
ETC:	electron transport chain
FCS:	fetal calf serum
GABA:	γ -aminobutyric acid
GAPDH:	glyceraldehyde-3 phosphate dehydrogenase
GDNF:	glial cell line-derived neurotrophic factor
GFR α :	GDNF-family receptor α
GPI:	glycosyl phosphatidylinositol
HRE:	hypoxia response element
Hsp 27:	heat shock protein 27
IEGs:	immediate early genes
IGF:	insulin-like growth factors
IASP:	International Association for the study of Pain
JNK/SAPK:	c-jun N-terminal kinase/stress-activated protein kinase
MAP:	microtubule associated protein
NRTN:	neurturin
NGF:	nerve growth factor
NGS:	Normal Goat Serum
NT-3:	neurotrophin-3

NT-4:	neurotrophin-4
NT-5:	neurotrophin-5
NF- κ B:	nuclear factor – kappa B
PBS:	phosphate buffered saline
PI:	propidium iodide
PI3K:	phosphoinositide-3-kinase
PKB:	protein kinase B
PKC:	protein kinase C
PN:	peripheral neuropathy
PSPN:	persephin
PT:	mitochondrial permeability transition pore
SEM:	standard error of the mean
SOD1:	superoxide dismutase 1
STZ:	streptozotocin
Trk:	tropomyosin-related kinase
TUNEL:	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VDCC:	L- type voltage dependent calcium channels
VEGF:	vascular endothelial growth factor

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IV Abstract

Peripheral neuropathy is a common complication of diabetes that can affect 50% of the patients. It predisposes the patient to several physiological problems in the lower limbs, which may lead to gangrene and even amputation. Due to its widespread prevalence and serious complications it is important to understand the pathogenesis of diabetic neuropathy, so appropriate therapies can be developed.

In this study the pathogenesis of diabetic peripheral neuropathy was investigated in both an in vitro and ex vivo model for this disorder. The effect of hyperglycemia on DRG neurons was investigated by measuring the neuronal stress response, measured by the level of ATF3, neurite outgrowth and neuronal viability. Both models showed an increase in the ATF3-level and a decline in neurite outgrowth. There were no significant changes in the number of apoptotic cells in diabetic rats, compared to control rats. These results indicate that hyperglycemia is indeed a key player in the development of diabetic neuropathy. In the second part of the study the beneficial effects of growth factors was documented. Therefore the effect of Enovin, GDNF and VEGF on the neuronal stress response was investigated. Both the in vitro and ex vivo model for diabetic neuropathy showed that all three growth factors can reduce neuronal stress. Furthermore, addition of ENV, GDNF and VEGF increased the overall neurite outgrowth.

This research indicates that all tested growth factors have a protective effect on hyperglycemia-induced changes in cultures of DRG neurons. Therefore these compounds can be considered as possible candidates for the treatment of diabetic peripheral neuropathy. However, further research must be conducted to test the safety and tolerance of these compounds before they can be applied for the treatment of this disorder in human patients.

IV Abstract

Perifere neuropathie is een veel voorkomende complicatie van diabetes, bijna de helft van de patiënten krijgt er mee te maken. Neuropathie kan, vooral in de onderste ledematen, een scala aan klachten veroorzaken, van hinderlijke gevoelsstoornissen tot ernstige zweren en zelfs amputatie. Door de hoge prevalentie en de ernstige complicaties is het belangrijk om de pathogenese van diabetische neuropathie te begrijpen, zodat doeltreffende therapieën ontwikkeld kunnen worden.

In deze studie werd de pathogenese van diabetische perifere neuropathie onderzocht in zowel een in vitro als een ex vivo model voor deze stoornis. Het effect van hyperglycemie op DRG neuronen werd onderzocht door het meten van de neuronale stress response, gemeten door het niveau van ATF3, de uitgroei van neurieten en neuronale viabiliteit. In beide modellen was een stijging in de neuronale stress respons en een daling in de uitgroei van neurieten zichtbaar. Er waren echter geen verschillen in het aantal apoptotische cellen in diabetische ratten vergeleken met controle ratten. Deze resultaten wijzen op de betrokkenheid van hyperglycemie in het ontstaan van diabetische perifere neuropathie. In het volgende deel van de studie werden de mogelijke protectieve effecten van groeifactoren onderzocht. Hiervoor werd het effect van Enovin, GDNF en VEGF op de neuronale stress respons onderzocht. In beide modellen werd duidelijk dat alle groeifactoren de neuronale stress response kunnen verminderen. Verder blijkt dat toevoeging van Enovin, GDNF, en VEGF leidt tot een sterkere uitgroei van neurieten.

Uit dit onderzoek blijkt dat alle groeifactoren een beschermend effect hebben op de culturen van DRG-neuronen. Daardoor kunnen deze componenten beschouwd worden als mogelijke kandidaten in de behandeling van diabetische perifere neuropathie. Verder onderzoek is echter nodig om de veiligheid en de tolerantie van deze componenten te testen alvorens deze ingezet kunnen worden in de behandeling van deze stoornis in diabetische patiënten.

1.Introduction

Pain is an essential part of the body's defence system: it warns the central nervous system that harm is being done. The definition of pain, defined by the International Association for the study of Pain (IASP), is different from that of nociception. The latter refers to the neurophysiologic manifestations generated by noxious stimuli while emotional experience plays an important role in pain. Pain that arises suddenly in response to a specific injury and disappears when this injury resolves is called acute pain. Chronic pain, on the other hand, persists for an extended period of time (1).

1.1 Nociception

The process of nociception starts by activation of peripheral nerve fibers in response to tissue damage or inflammation. These nerve fibers are responsible for the generation of nerve impulses, which are conducted into the central nervous system (CNS). There the sensation is transferred to the level of consciousness (2).

1.1.1 Peripheral receptors

Pain is initiated when peripheral receptors, also called nociceptors, become activated. Activation can occur by mechanical, thermal or chemical stimuli (1).

1.1.2 Afferent pathways

After activation of nociceptors, first-order fibers transport the signal to the spinal cord. These fibers are classified as C-, A δ - or A β -type fibers. A δ -fibers are thinly myelinated and propagate specific information with high intensity and short latency. They induce a quick sensation of first phase or acute pain and thereby trigger withdrawal actions. C-fibers, with a slower conduction rate, are unmyelinated. They carry thermoreceptors, mechanoreceptors of low threshold and specific receptors for substances such as potassium, acetylcholine and histamine.

The A β -fibers, in contrast to the previous two fibers, are well myelinated but normally not involved in the propagation of noxious stimuli. These fibers carry mechanoreceptors.

The afferent information from the fibers reaches the dorsal root ganglion (DRG), a nodule on the dorsal root that contains the cell bodies of the sensory neurons. They transmit the sensory information further to the dorsal horn of the spinal cord and finally into the brain (1).

1.2 Neuropathic pain

Neuropathic pain is a chronic condition that is caused by an injury to the nervous system. A change of the neuron itself, its supporting glial cells and the interacting environment can lead to a long-lasting dysfunction of the neuron and thereby result in neuropathy. Inflammation within the nerve or ganglion for example may introduce new chemical messengers that alter the function, chemistry and even the survival of the neurons. There is currently no treatment to prevent the development of neuropathic pain or to control it (3,4).

The condition is called central neuropathy when the lesion occurs within the central nervous system. Peripheral neuropathy refers to damage or dysfunction to the nerves of the peripheral nervous system. The latter is the most common and will therefore be emphasized in the current work (5).

1.2.1 Peripheral neuropathic pain

IASP defines peripheral neuropathic pain as pain resulting from a primary lesion or dysfunction of the peripheral nervous system. Feelings of electric shock, burning and tingling are the most common complaints of patients suffering peripheral neuropathy. These adjectives are particularly valuable in discriminating patients with neuropathic pain. It is a common disorder: the prevalence in the United States has been estimated to be 0.6% but precise figures are difficult to obtain (6).

Peripheral neuropathy can arise from different etiologies including genetic factors, chronic disease, environmental toxins, alcoholism, or side effects of certain medications (7, 8).

1.2.1.1 Diabetic neuropathies

Diabetic polyneuropathy is the most common complication of diabetes mellitus and is the major cause of morbidity and mortality in diabetic patients. This disorder affects both the small- and large-fibers of the peripheral nervous system leading to a spectrum of structural changes in the peripheral nerve, like axonal degeneration, paranodal demyelination and loss of myelinated fibers. The earliest signs of diabetic neuropathy, associated with small-fiber dysfunction, are loss of thermal sensitivity and diminished sensation of touch in the lower limbs. Due to this decreased tactile and pain perceptions, injuries to the feet are often undetected which can lead to severe side-effects such as foot ulcerations, gangrene and even amputation. The later state of diabetic neuropathy, when large-fibers are affected, is characterized by loss of vibration and proprioceptive perception, weakness and depressed tendon reflexes. In a study of Pirart the prevalence of diabetic neuropathy reached approximately 45% after 25 years of diabetes. This means that in the United States seven million patients are suffering from this syndrome (9). Despite these numbers, only recently the major contributing pathogenic mechanisms have been identified. Four major pathways that can lead to hyperglycemia-induced neuronal damage have been discovered.

The first mechanism is an increased flux through the polyol pathway, which focuses on the enzyme aldose reductase. Normally the function of this enzyme is to reduce toxic aldehydes to inactive alcohols. However, in hyperglycemic conditions aldose reductase converts the excess glucose to sorbitol. Sorbitol is later oxidized to fructose. The latter process consumes NADPH, an essential cofactor for the reduction of glutathione. The reduced form of glutathione is an important anti-oxidant enzyme. The decreased levels of NADPH leads to a decreased production of reduced glutathione and shift the balance to more oxidative stress. This can harm the DNA, proteins and lipids of the cells. Mitochondrial DNA in particular is very susceptible to oxidative stress. This can ultimately lead to an altered cell survival and cell energy regulation, which is extremely important in high-energy requiring neurons. Alterations of the lipids and proteins inhibit normal functioning of the cells. Axonal transport, for example, can be diminished. This leads to a decreased neuronal survival (10-13).

Another way to cause neuronal damage is an increased intracellular production of advanced glycation end product (AGE) precursors. An important source for AGE to arise is the auto-oxidation of glucose to glyoxal. Production of AGE precursors can damage the cells by three general mechanisms. First, AGE can directly modify intracellular proteins including proteins involved in gene transcription. These precursors can increase the expression of angiotensin-2, a factor that has been implicated in capillary regression. This will soon lead to hypoxia, a contributing factor in the pathogenesis of diabetic peripheral neuropathy. Second, the AGE precursors can also diffuse out of the cell and modify extracellular matrix molecules, which can interfere with the growth-promoting sequence in the A chain of the laminin molecule. This event reduces neurite outgrowth. The final possibility is the potential of these precursors to modify circulating proteins in the blood such as albumin. These can bind to AGE-receptors which leads to the activation of the transcription factor NF- κ B and ultimately result in the production of inflammatory cytokines and growth factors. These factors can also cause vascular pathology and ultimately diabetic peripheral neuropathy (11-13).

The third mechanism is activation of protein kinase C (PKC). Hyperglycemia-induced activation of PKC has several consequences like blood flow-abnormalities, an increased oxidative stress and a pro-inflammatory gene expression (11). These mechanisms are, as discussed in the other pathways, devastating for the neurons.

The fourth mechanism is the increased flux through the hexosamine pathway. This pathway diverts glucose into uridine diphosphate (UDP) N-acetyl glucosamine. This compound can bind to residues of transcription factors and leads to a change in gene expression with potential pathological consequences. For example, increased modification of transcription factor Sp1 results in increased expression of transforming growth factor- β and plasminogen activator inhibitor-1, which have negative influences for diabetic blood vessels (11).

These four pathways all reflect to a single hyperglycemia-induced process: overproduction of superoxide by the mitochondrial electron-transport chain (ETC, figure 1.1).

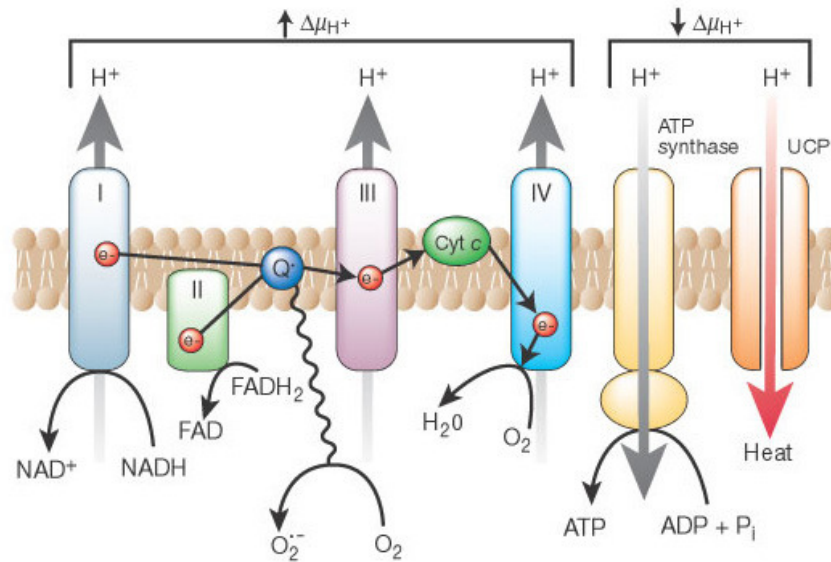


Figure 1.1: the electron transport chain. The high mitochondrial inner membrane potential inhibits electron transport at complex III, increasing the half-life of free-radical intermediates of coenzyme Q. This reduces oxygen to superoxide (11).

Increased hyperglycemia-derived electron donors generate a high mitochondrial membrane potential by pumping protons across the mitochondrial inner membrane. As a result the voltage gradient across the mitochondrial membrane increases until a critical threshold. This event inhibits the electron transport at the third complex and causes a reduction of oxygen into superoxide, one of the main causes of oxidative stress. This mitochondrial superoxide causes a decrease in the activity of the key glycolytic enzyme glyceraldehydes-3 phosphate dehydrogenase (GAPDH). Ultimately, this leads to activation of the polyol pathway, increased intracellular AGE formation, activation of PKC and increased flux through the hexosamine pathway (13).

Overall, hyperglycemia has emerged as a major risk factor for the development of diabetic neuropathy. This can affect the peripheral sensory nerves through several mechanisms (figure 1.2). Eventually these mechanisms lead to a reduced bioavailability of neurotrophic factors, reduced activity of the Na⁺K⁺ ATPase activity and vasoconstriction. Degradation of the axonal structure is the inevitable consequence.

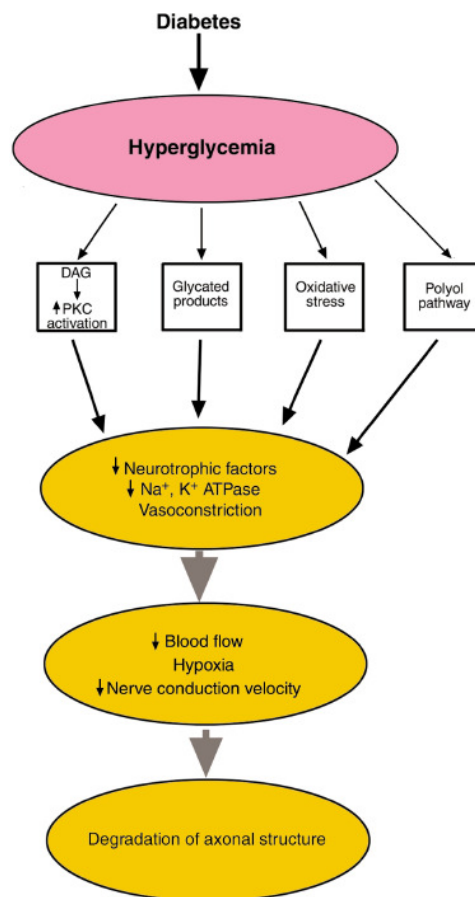


Figure 1.2: the pathogenesis of diabetic neuropathy. The key factor in the pathogenesis of diabetic neuropathy is hyperglycemia, which decreases, by the pathways described above, the neurotrophic support and the activity of the Na⁺, K⁺-ATPase activity. High-glucose levels also lead to vasoconstriction. This leads to hypoxia and a diminished blood flow and nerve conduction velocity. These effects will degrade the axonal structure (adapted from Veves et al. 2001).

1.2.1.2 Treatment of peripheral neuropathy

Despite the prevalence and the devastating effects of peripheral neuropathy, the treatment options are limited. Often therapy is instituted with the intentions to ameliorate symptoms and

to prevent the progression of the neuropathy, rather than to reverse the underlying causes of this pain. Many of the symptoms of neuropathic pain seem to be provoked by alterations in the peripheral nervous system. The peripheral nerves become more excitable and this can lead to ongoing, spontaneous activity. The source of these changes is an altered expression in ion channels. There is an increase in the expression of sodium- and calcium channels in DRG neuronal cell bodies and in nociceptive neurons of the spinal cord and dorsal horn. Many modern drugs block these channels and thereby diminish the action potentials. This ultimately leads to relieve of peripheral nerve pain. Commonly used drugs in this category are gabapentin and carbamazepine (14, 15).

Carbamazepine is a sodium channel blocker. At therapeutic levels it slows the recovery rate of voltage gated sodium channels, which limits repetitive firing. About 70% of patients with neuropathy benefit from this treatment (16).

Gabapentin is a structural analogue of the neurotransmitter γ -aminobutyric acid (GABA). It works by binding to a subtype of L-type voltage-dependent calcium channels (VDCC), which reduces calcium channel currents. It has proven efficacy in patients with post herpetic neuralgia and diabetic neuropathy (17, 18).

1.3. Neurotrophic factors

Neurotrophic factors are natural proteins that promote the growth and survival of neurons. They are released by target tissues of responsive neurons and bind to specific receptors on the neuron. After binding, these neurotrophic factors are retrogradely transported to the cell body where they regulate gene expression through second-messenger systems. The current knowledge of growth factors and their relationship with neuropathy suggest that impaired neurotrophic support is frequently involved in neuronal dysfunction.

The most important neurotrophic factors regarding neuropathy are nerve growth factor (NGF), brain-derived growth factor (BDNF), neurotrophin-3 (NT3) and the glial cell line-derived neurotrophic factor (GDNF) family because these have been studied most extensively with regard to painful peripheral neuropathy (19). Increasing attention has been paid to the vascular endothelial growth factor (VEGF) in neuroprotection.

1.3.1 Neurotrophins

Neurotrophins are a family of structurally related neurotrophic factors, consisting of five members: NGF, BDNF, NT-3, neurotrophin-4 (NT-4) and neurotrophin-5 (NT-5). NT-4 and NT-5 were discovered separately but it is now accepted that they are interspecies variants of the same protein and became referred to as NT-4/5 (20).

During neuronal development NGF is essential for the survival of DRG neurons. However, in adults the main function of NGF is to maintain physiologic neuropeptide levels, for instance the level of substance P. This neurotrophic factor is synthesized and released by target tissues, including skeletal muscle and skin, and binds to its receptor on the nerve terminal (21, 22). Receptors for NGF include the high affinity receptor tropomyosin-related kinase (Trk), in this case TrkA, and the low-affinity nerve growth factor receptor p75. Binding of NGF to TrkA results in receptor dimerization and phosphorylation, which leads to activation of phosphoinositide-3-kinase (PI3K) and MAP kinase pathways. Both pathways regulate neuronal survival and neurite outgrowth. The role of the p75 receptor is still to be elucidated. As a result of the higher NGF-levels in damaged tissue, the level of neurotransmitters also increases. This causes a higher response of a nerve on a stimulus that is normally painful. The latter is also called hyperalgesia. This indicates that blocking of NGF action might be useful for some types of neuropathy. However, the opposite strategy could be useful in preventing or reversing the neuronal disorder. The particular nerve disorder and patient subset would dictate whether a pro- or anti-NGF strategy is appropriate (21, 23).

BDNF supports medium-sized fiber sensory neurons involved in mechanical pressure sensation. It also promotes the survival of motoneurons. NGF can increase the production of BDNF. The mechanisms of action in spinal neurons involve binding to the high-affinity receptor TrkB. This is followed by phosphorylation of intracellular targets such as the NMDA receptors. These receptors are important in spinal cord excitability and could explain central sensitization. In pathological states the production of BDNF increases. Thus, the best treatment in pain states would be blocking of the BDNF action (21, 24).

NT-3, the third member of the neurotrophins superfamily, supports large neurons of dorsal root ganglia that mediate proprioception. NT-3 binds to the high-affinity TrkC receptor and promotes peripheral nerve generation. Some experiments show a decrease in axonal transport of NT-3 and TrkC mRNA expression in DRG neurons of diabetic rats. These results suggest that NT-3 could be helpful in the battle against peripheral neuropathies (19, 22).

NT-4/5 has similar effects compared with BDNF with regard to responsive neuronal populations because they share the same receptor, the high-affinity TrkB receptor. In diabetic nerves mRNA of NT-4/5 is decreased but more experiments must be carried out to completely clarify the role of NT-4/5 in pathological conditions (19).

1.3.2 Glial cell line-derived neurotrophic factor family

Members of the glial cell line-derived neurotrophic factor (GDNF) family are widely expressed throughout the central and peripheral nervous system as well as in non-neuronal tissues. In animal models of Parkinson's disease GDNF protects dopaminergic neurons and rescues motor neurons. Hope was raised that GDNF could serve as a therapeutic agent in neuropathies (19).

The GDNF-family consists of four molecules: GDNF, neurturin (NRTN), persephin (PSPN) and artemin (ARTN). The latter is also known as neublastin or enovin. All these family members signal through the RET receptor tyrosine kinase. This receptor is only activated when a GDNF-ligand and its proper GDNF-family receptor α (GFR α) bind to it. The GFR α is linked to the membrane by a glycosyl phosphatidylinositol (GPI) anchor. There are also four members of this co-receptor: GFR α 1, GFR α 2, GFR α 3 and GFR α 4 that preferentially interact with GDNF, neurturin, artemin and persephin (23,25). Binding of a GDNF-ligand and its proper GFR α to the extracellular domain of RET leads to activation of the intracellular tyrosine kinase domain and thereby phosphorylation of the receptor. Once the RET-receptor becomes phosphorylated, the tyrosine residues in the intracellular domain serve as high-affinity binding sites for various intracellular signaling proteins in target cells (figure 1.3). These sites can eventually trigger a cascade of intracellular events that lead to neuroprotection.

Addition of GDNF seems an attractive therapy for the treatment of neurodegenerative disorders, including neuropathies. However, *in vivo* studies show that GDNF elicits side effects that are not acceptable in the clinic such as weight loss and allodynia. The latter refers to pain caused by a stimulus that is normally not painful (19, 25).

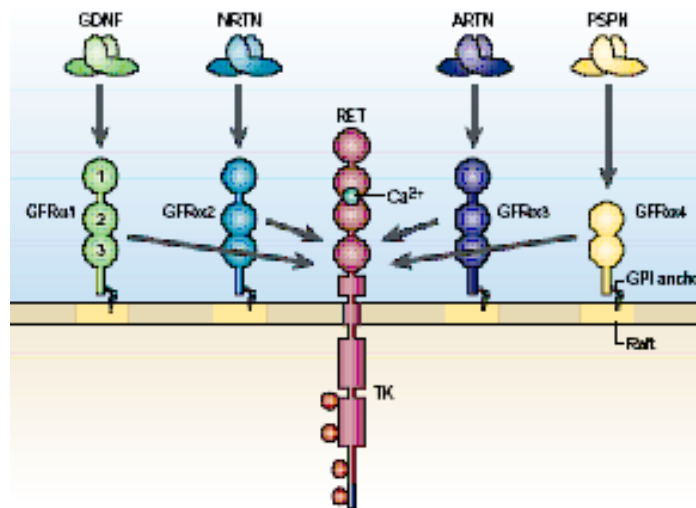


Figure 1.3: GDNF family and receptor interactions. GDNF-family ligands activate RET tyrosine kinase (TK) by first binding their cognate GDNF-family receptor α (GFR α) (25).

1.3.3 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a prime regulator of endothelial cell proliferation, angiogenesis and vascular permeability. The activity of VEGF is mediated by the high-affinity tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR) and VEGFR-3 (Flt4). Hypoxia is a major stimulus for upregulation of VEGF expression, which occurs through the hypoxia response element (HRE) in the promoter of the VEGF gene. The pathways converge at MAPK-activation and ultimately lead to endothelial cell proliferation, migration and survival (26, 27).

However, there are several lines of evidence indicating that VEGF and its receptors have influences on the nervous system. For example, VEGF stimulated axonal outgrowth and increased neuronal survival in cultures of DRG neurons. In addition, inhibition of VEGFR-2 signaling blocked axonal outgrowth in response to VEGF (28).

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is characterized by a selective loss of motoneurons in the brain and spinal cord. Mutations in the superoxide dismutase 1 (SOD1) gene cause motodegeneration in humans and mice. Therefore, mice expressing the human SOD1 with a Gly93 mutation have become the standard model for evaluation of drug candidates for this disease. Previous studies reported that low levels of VEGF cause ALS-like motoneuron degeneration in this gene-targeted mouse. In addition, intramuscular transfer of the VEGF gene delays onset of the disease and prolongs the survival of the mice. These observations indicate that VEGF is an attractive candidate for ALS treatment (29,30).

Hypoxia in the peripheral nerves is a contributing factor in the pathogenesis of diabetic neuropathy. The resulting hypoxia enhances VEGF levels in neurons and Schwann in experimental diabetes animal models. VEGF gene transfer was tested as a candidate to ameliorate the onset or progression of diabetic neuropathy with several positive results. First, VEGF gene therapy has shown to prevent or reverse the establishment of axonal loss and myelin degeneration that was observed in untreated animals. Second, it was reported that VEGF stimulated the migration and prevented the hypoxia-induced apoptosis of Schwann cells in vitro, which expressed VEGF receptors. These results suggest that VEGF, in addition to restoring blood flow by inducing angiogenesis, may directly promote the survival of peripheral nerve cells. Such characteristics would make VEGF an ideal agent for preventing or restoring nerve dysfunction in diabetic peripheral neuropathy (31,32).

1.3.4 Intracellular signal transduction cascades

Insulin, insulin-like growth factors (IGFs) and neurotrophins mediate growth and survival responses in neurons via phosphoinositide 3-kinase (PI3K) -dependent regulation of Akt. This protein is also known as protein kinase B (PKB).

Binding of insulin or neurotrophic factors to their receptors activates PI3K. This event triggers activation of Akt leading to translocation of this protein to the cytosol and the nucleus. In the nucleus it can phosphorylate its downstream target cAMP response element binding protein (CREB).

Akt-dependent activation of CREB can elevate hexokinase II expression because hexokinase II has promoter sequences for CREB. It has been hypothesized that Akt can directly modulate hexokinase II activity in the cytosol. Elevated activity of this element will stimulate increased flux of glucose through the glycolytic pathway providing elevated levels of ATP, which in turn contributes to the energy requirements of neurons (figure 1.4, 33).

The other mechanism of neuroprotection via Akt is the prevention of apoptosis. Akt can phosphorylate the pro-apoptotic protein Bad leading to its downregulation. Furthermore, it has been suggested that Akt can lead to an upregulation of Bcl-2. The promoter region of the anti-apoptotic Bcl-2 contains an ATF/cyclic AMP response element (CRE)-site. Binding of CREB to this site, under the influence of Akt, will lead to an increased Bcl-2 expression (34).

Overall Akt promotes cell survival via an upregulation of ATP and the anti-apoptotic Bcl-2 protein. In neurons, loss of neurotrophic support and the associated deficits in Akt-signaling leads to deprivation of the ATP-stores and promotion of apoptosis. This results in devastating effects for the neurons and is therefore a contributing factor in the pathogenesis of diabetic neuropathy.

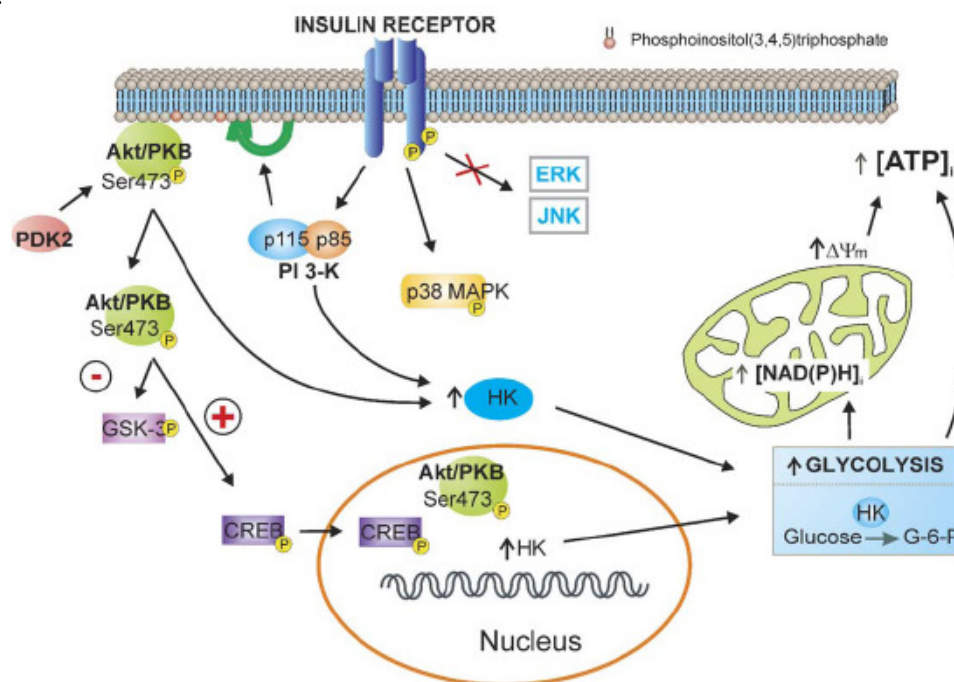


Figure 1.4: the Akt pathway. Insulin signals through the insulin receptor to activate phosphoinositide 3-kinase (PI3K) that in turn triggers phosphorylation of Akt. Akt elevates the levels of hexokinase II. Increased hexokinase activity will drive increased flux of glucose through the glycolytic pathway providing elevated levels of ATP. The outcome is an insulin-dependent augmentation of ATP synthesis that contributes to the energy requirement for driving axon regeneration (33).

1.4 Neuronal stress

Recent studies about gene expression following nerve injury have identified several immediate early genes (IEGs) as key players in the response to axonal injury. IEGs regulate the expression of specific target genes that have important functions in the neuronal response to injury. Among the IEGs induced in the DRG neurons, activating transcription factor 3 (ATF3) and c-jun are the most important (35).

1.4.1 Activating transcription factor 3

Activating transcription factor 3 (ATF3) is a member of the ATF/CREB family of transcription factors. Members of this family bind to the CRE site. ATF3 is induced in a variety of cell types by many stress signals indicating that ATF3 is a key regulator in cellular stress responses. In physiological conditions it is not detected in the neuronal cell. However, it is highly expressed in response to nerve injury. This makes ATF3 a unique nerve injury marker. The ATF3-homodimer is known to function as a repressor, whereas the heterodimer, for instance ATF3/c-jun, functions as an activator of specific promoters (4,34-39).

1.4.2 C-jun

C-jun is another member of the CREB/ATF family. Similar to ATF3, c-jun is a stress-inducible factor. It is activated by c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and induces apoptosis in many types of cells, including neurons (35,39-41).

1.4.3 ATF3/c-jun heterodimer

ATF3 and c-jun are both stress-inducible transcription factors and can interact with each other by forming a heterodimer. The co-expression of ATF3 with c-jun suggest that interaction between these transcription factors may be important for controlling the program of gene expression necessary for neuronal survival and axonal regeneration. The combination of ATF3 and c-jun induces the anti-apoptotic heat shock protein 27 (Hsp 27), which activates Akt. This may lead to survival of neurons and promotion of neurite elongation in the cells (35, 37-41).

1.5 Objective of the study

Neuropathy is a common disorder; approximately 3.8 million individuals in the United States have neuropathic pain. It is a major cause of morbidity and mortality. Not only does it cause painful syndromes in a subset of patients, it also affects the quality of life. Due to its widespread prevalence and serious complications, it is important to understand the pathophysiologic mechanisms leading to the development of neuropathy and eventually translate this in a more specific treatment.

The first goal of the study was to further unravel the underlying pathogenesis of peripheral neuropathy, particularly diabetic neuropathy. To investigate this, DRG neurons were isolated from control rats and treated with glucose as an *in vitro* model for diabetic neuropathies. In the *ex vivo* model DRG neurons from healthy control rats were isolated and compared to DRG neurons from diabetic rats. The neuronal stress response, neuronal viability and neurite outgrowth were investigated to study the effect of hyperglycemia on these DRG cultures.

The other goal of the study was to investigate the potential of growth factors to protect DRG neurons against hyperglycemia-induced changes. This was done by using an *in vitro* and *ex vivo* model for diabetic neuropathy. The neuronal stress response and neurite outgrowth were tested to investigate the beneficial effects on DRG neurons and hopefully find potential candidates for future therapies.

2. Material & Methods

For this study DRG neurons from male rats were isolated because these neurons are the target tissue in the pathophysiology of peripheral neuropathy. The sensory perikaryons of the peripheral nervous system are located within the DRG. Furthermore, these neurons are less protected from toxins in the circulation and are therefore sensitive to toxic insults.

2.1 Rat models

In this study male Sprague- Dawley rats (Harlan, Netherlands) were used. They had access to water *ad libitum*. In a subgroup of rats diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 65 mg/kg i.e. 6.5 mg/ml and 1 ml/100 g rat, Sigma, USA) to animals fasted overnight (starvation for 20-24 hours). STZ is a chemical that is particularly toxic to the insulin producing beta cells of the pancreas. Therefore it is a good representation of type I diabetes. Blood samples, for measurement of glucose, were taken from the tail vein 48 hours after STZ-injection. Only rats with a blood glucose level above 500 mg/dl were considered diabetic and were used for further experiments. Control rats received a buffer solution instead.

2.2 Isolation of dorsal root ganglion neurons

Rats were killed by inhalation of carbon dioxide (CO₂). Immediately thereafter the DRG neurons were dissected from the spinal column (figure 2.1) and collected in a PBS solution (-Ca²⁺, -Mg²⁺) enriched with 1 g/l glucose. After the isolation, the ganglia were enzymatically dissociated by incubating them, at 37°C, for 45 minutes in medium containing 0.5% collagenase (Roche Diagnostics, Germany), followed by 30 minutes incubation in 0.25% Trypsin (Gibco, UK) in PBS. Next, the ganglia were collected in Dulbecco's Modified Eagle Medium (DMEM; Gibco, UK) supplemented with fetal calf serum, non-essential amino acids, sodium bicarbonate and L-glutamine. In this medium the cells were mechanically dissociated into single cells by titration through flame-constricted Pasteur pipettes of decreasing tip-diameter.

The cells were then placed in a Petri dish, previously coated with fetal calf serum (FCS, Hyclone, USA) for 90 minutes at 37°C, to obtain a more pure cell culture. The coating allows non-neuronal cells to adhere faster to the dish while the neuronal cells stay in suspension. Finally, the DRG neurons were plated into poly-L-lysine (Sigma, USA) coated well plates in Neurobasal medium (Gibco, USA) to meet the special requirements of neuronal cells. The medium was enriched with B27 supplement (Gibco, UK), which contains substances necessary for growth and long-term viability of neurons. The neurons that were intended for analysis of the neuronal stress response were plated into 96-well plates. Neurons for determination of the neuronal viability and neurite outgrowth were plated into 12-well plates.

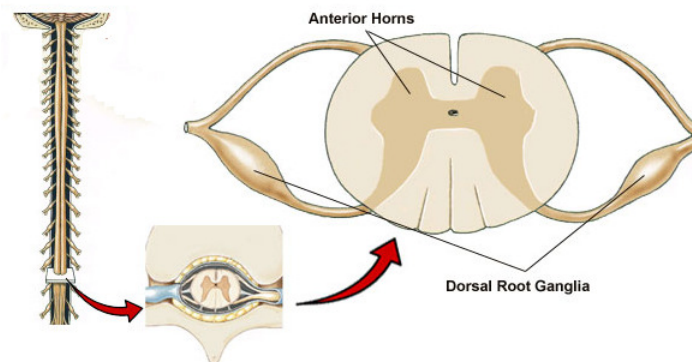


Figure 2.1: dorsal root ganglia. This is a nodule on a dorsal root that contains cell bodies of neurons in afferent spinal nerves.

2.3 Fixation of the cells

DRG neurons were fixed for 10 minutes in 0.5% Triton X100 (Sigma, USA), to permeabilize the cells, and 0.5% Glutaraldehyde (Fluka Chemie, Switzerland) in PHEM buffer (2 liter PHEM buffer contains 3.02 g PIPES, 1.52 g EGTA, 0.38 g $MgCl_2$, 1 l Double Hanks buffer containing 2.0 g glucose, 0.8 g KCl, 16 g NaCl, 0.12 g KH_2PO_4 and 0.7 g $NaHCO_3$ in distilled water). After washing the cells with PHEM, the cells were incubated with 0.5% Triton X100. Addition of 1 mg/ml $NaBH_4$ dissolved in PHEM buffer, to reduce the free aldehyde groups, followed after another wash with PHEM. Finally, the cells underwent a last wash in PHEM buffer. The neurons that were intended for analysis of the neuronal stress response were fixed after 24 hours. The neurons that were plated in 12-well plates were analyzed for neuronal viability after three days. Afterward these plates could be fixed and neurite outgrowth was measured.

2.4 Immunocytochemistry

Immunocytochemistry begins with incubation of the cells for 30 minutes in PBS/Bovine Serum Albumin (BSA, Sigma, USA) buffer containing 5% Normal Goat Serum (NGS, Sigma, USA).

For measurement of the neuronal stress response, the cells were dual-labeled with a polyclonal Anti-ATF3 antibody (Santa Cruz Biotechnology, USA) at 1:800 dilution in PBS/BSA and a monoclonal SMI32 antibody (Sternberger Monoclonals Incorporated, USA) at 1:1000 dilution in PBS/BSA. ATF3 identifies individual neurons undergoing cell damage. SMI32 recognizes non-phosphorylated neurofilament proteins. In this way SMI32 can visualize neuronal cell bodies, dendrites and axons and serves as a neuronal marker. Following overnight incubation at room temperature the cells were washed four times with PBS/BSA. Afterwards, the neurons were incubated for one hour at room temperature with Alexa Fluor 555 Goat Anti-Rabbit IgG (Invitrogen Molecular Probes, USA) and Alexa Fluor 488 Goat Anti-Mouse IgG (Invitrogen Molecular Probes, USA). The cells were washed with PBS/BSA. The nuclei of the cells were counterstained by addition of 4', 6-Diamidino-2-phenylindole (DAPI, Sigma, USA).

For determining neurite outgrowth, neurons were labeled with one antibody instead of two. The primary antibody was polyclonal Anti-Tau (Invitrogen Molecular Probes, USA) at 1:2000 dilution in PBS/BSA. Tau is one of the most abundant microtubule associated proteins and is important for microtubule stability, axon elongation and axon structure. It is a neuron specific antibody so it was used to visualize the neuronal cell body and the neurites. After one night incubation and washing of the cells with PBS/BSA the cells received their secondary antibody. This was Alexa Fluor 555 in which the cells were incubated for one hour at room temperature.

Finally, the cells were also labeled with calcein (Invitrogen Molecular Probes, USA), Propidium Iodide (PI, Invitrogen Molecular Probes, USA) and Hoechst (Molecular Probes Europe BV, Holland) to measure neuronal viability. Calcein, which can be transported into living cells, is used to stain viable cells. Propidium iodide is membrane impermeant and generally excluded from viable cells. It can bind to the DNA of cells and is therefore used to distinguish normal nuclei from the apoptotic or necrotic ones.

Hoechst can also bind to the DNA of the cells and is therefore generally used a marker for intact nuclei. However, the nucleus of an apoptotic cell is more condense. When Hoechst binds to an apoptotic nucleus, it gives a very bright bleu staining. For this labeling, the DRG neurons were first incubated for 5 minutes at 37°C with calcein, at 1:100 dilution in PBS, and PI at 1:5000 dilution in PBS. Afterwards, Hoechst at 1:1000 dilution in PBS was added.

2.5 Analysis

For the quantification of the ATF3 levels a fluorescence microscope, Zeiss Axiovert with 40X objective, was used using filtersets with the appropriate wavelengths to excite the individual fluorescent probes. The SMI32 labeled neurons were excited at 488nm and emit a green fluorescence, while the ATF3 labeled cells emit a red wavelength after excitation at 555nm. DAPI excites at 350nm, the UV-range, and emits a blue light. The ATF3-positive and ATF3-negative DRG neurons were counted. At least 100 neurons per well were counted. The percentage of ATF3-positive DRG neurons is a reflection of the neuronal stress.

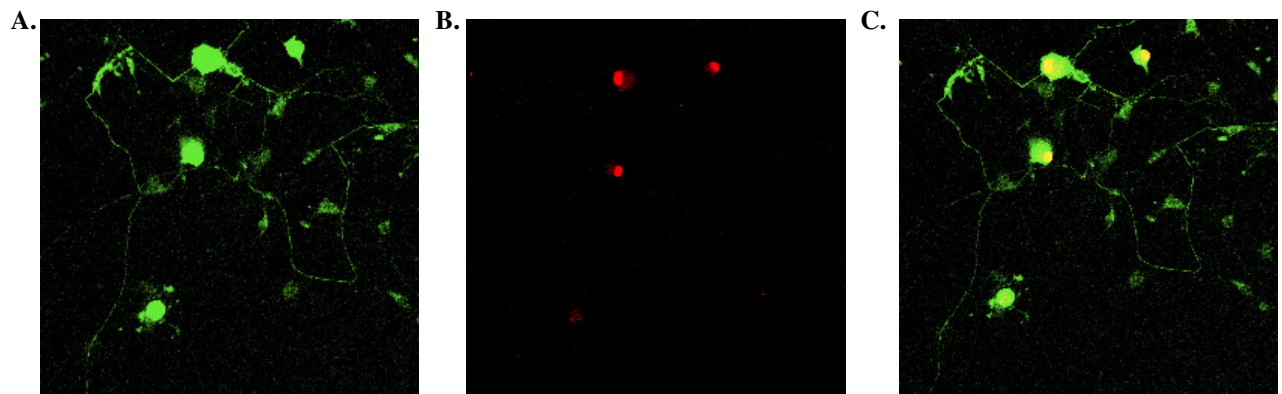


Figure 2.2: Examples of DRG neurons stained with ATF3 and SMI. Picture A shows neurons stained with SMI. Photograph B visualizes the neurons labeled with ATF3, to visualize the neuronal stress response. The last image shows an overlap of the first two pictures.

Neurite outgrowth was automatically analyzed by means of image analysis. With a confocal microscope, the Zeiss LSM510 with 10X objective, images of 10 randomly selected fields in every well were made. The quantification was done using image analysis. A dedicated software package identified cells and neurites. A report was generated that contained the position of each cell, the number of neurites attached to it and their respective length.

The neuronal viability was also measured by using a fluorescence microscope. For the visualization of the cells a Zeiss Axiovert 135 with 40X objective was used. Three filters were applied that let through radiation with the appropriate wavelengths to excite the fluorescent probes. The calcein labeled neurons were excited at 488nm and excite a green fluorescent light, while the PI-labeled cells were excited at 525nm and excite a red fluorescent light. The neurons that were labeled with Hoechst emit a blue light when excited at 350nm. The cells with a bright red nucleus were considered apoptotic.

2.6 Statistics

The results are shown as the mean \pm standard errors from n measurements. To see whether the mean values were significantly different, an unpaired student-t-test with equal variances was performed. A p-value less than 0.05 was considered to be statistically significant.

3. Results

The effect of hyperglycemia and growth factors on rat DRG neurons was analyzed by measuring the level of ATF3, neurite outgrowth and neuronal viability in the samples. These experiments were conducted in both an in vitro and ex vivo model for diabetic neuropathy.

3.1 Neuronal stress

In the first part of this research the effect of hyperglycemia and growth factors on the neuronal stress response in DRG neurons was investigated to clarify the pathogenesis of diabetic neuropathy.

3.1.1 Effect of glucose on neuronal stress

To study the in vitro effect of glucose on neuronal stress, cultured DRG neurons of all levels were exposed to different hyperglycemic conditions. Concentrations of 10 mM, 20 mM, 30 mM, 40 mM, 50 mM or 80 mM glucose were added to the medium of the cells, which already contained 30 mM glucose. After 24 hours the cells were fixed and labeled for ATF3. Neuronal stress was measured by counting the number of ATF3-positive and ATF3-negative DRG neurons in the cultures.

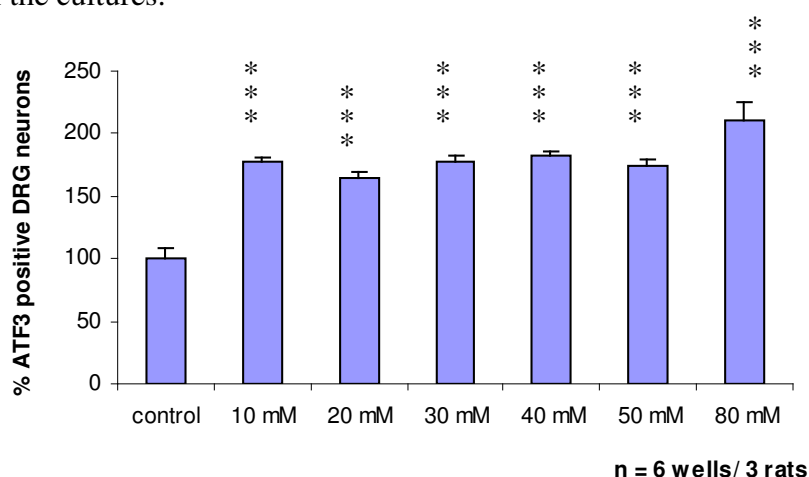


Figure 3.1: Effect of glucose on neuronal stress in DRG neurons. All DRG neurons from control rats were isolated and cultured in 96-well plates. The cells were treated with different concentrations of glucose for 24 hours. After fixation and labeling, the percentage of ATF3-positive DRG neurons was determined by counting the ATF3-positive and ATF3-negative neurons in each well. Shown is the mean of 6 wells from 3 rats \pm SEM (student t-test vs. control: *** $p < 0.001$).

The figure shows that increasing concentrations of glucose in the medium lead to an increase in the percentage of ATF3-positive DRG neurons when compared to control conditions. There is no real dose-response effect. The increase in the ATF3-level is visible when concentrations of 10 mM to 50 mM glucose are added to the medium of the cells. An addition of 80 mM glucose to the cells almost doubles the ATF3 expression. This indicates that chronic exposure of DRG neurons to high glucose concentrations leads to an increase in the ATF3-level. In the next in vitro experiments 80 mM glucose was used to induce neuronal stress.

3.1.2 Pharmacological modulation of glucose-induced neuronal stress

To evaluate the protective effect of growth factors on glucose-induced neuronal stress, cultured DRG neurons from control rats were pretreated with different concentrations of Enovin, GDNF and VEGF. After 4 hours 80 mM glucose was added to the culture medium for 24 hours to induce neuronal stress. Neuronal stress was determined by counting the percentage of ATF3-positive DRG neurons in the samples.

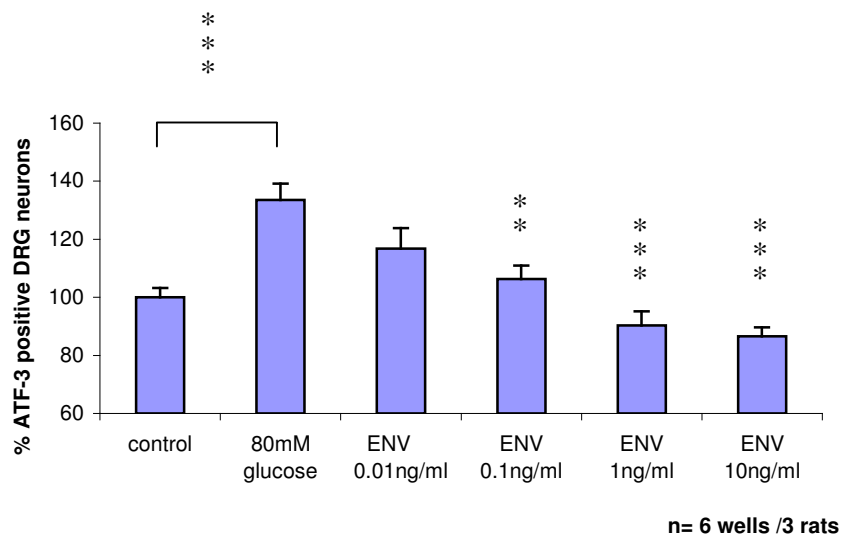


Figure 3.2a: *In vitro* effect of Enovin on glucose-induced neuronal stress in DRG neurons. DRG neurons from control rats were isolated and cultured in 96-well plates. The cells were pretreated for 4 hours with different concentrations of ENV (0.01 ng/ml, 0.1 ng/ml, 1 ng/ml and 10 ng/ml). Adding 80 mM glucose for 24 hours induced neuronal stress. Afterwards the percentage of ATF3-positive DRG neurons was determined by counting the ATF3-positive and ATF3-negative DRG neurons in each well. Shown is the mean of 6 wells from 3 rats \pm SEM (student t-test vs. 80 mM glucose: ** $p < 0.1$, *** $p < 0.01$).

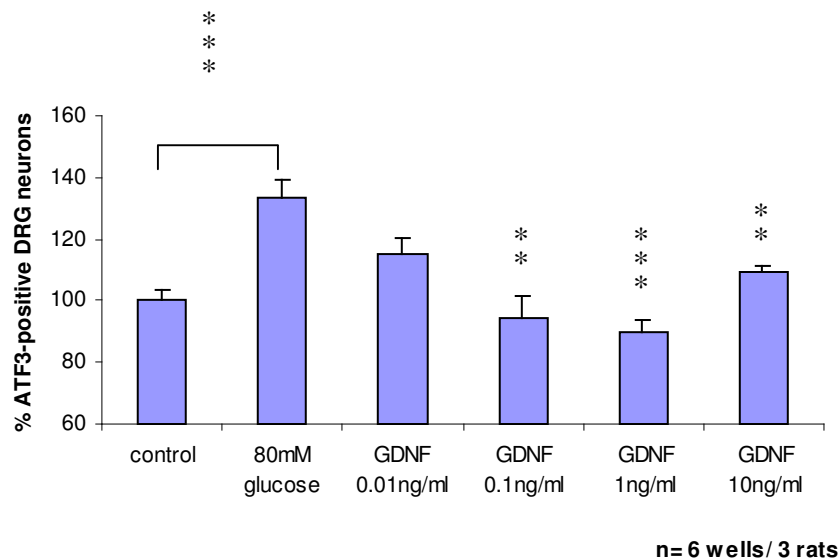


Figure 3.2b: *In vitro* effect of GDNF on glucose-induced neuronal stress in DRG neurons. DRG neurons, isolated from control rats, were plated in 96-well plates and pretreated for 4 hours with different concentrations of GDNF (0.01 ng/ml, 0.1 ng/ml, 1 ng/ml and 10 ng/ml). Afterwards, 80mM glucose was added to the medium of the cells for 24 hours to induce neuronal stress. The percentage of ATF3-positive DRG neurons was determined. Shown is the mean of 6 wells from 3 rats \pm SEM (student t-test vs. 80 mM glucose: ** $p < 0.1$, *** $p < 0.01$).

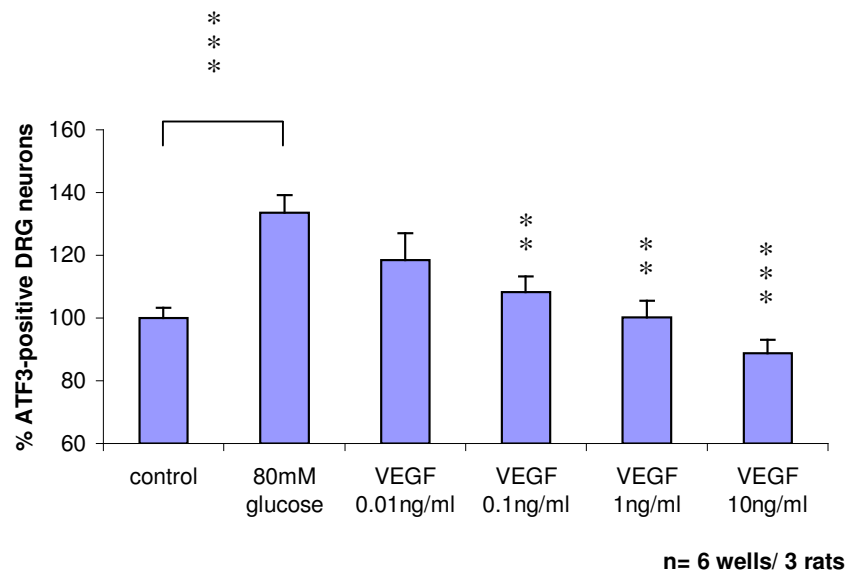


Figure 3.2c: *In vitro* effect of VEGF on glucose-induced neuronal stress in DRG neurons. Control DRG neurons were plated in 96-well plates. The cultured cells were pretreated with different concentrations of VEGF (0.01 ng/ml, 0.1 ng/ml, 1 ng/ml and 10 ng/ml). After 4 hours neuronal stress was induced by adding 80 mM glucose for 24 hours. Afterwards the percentage of ATF3-positive DRG neurons was determined. Shown is the mean of 6 wells from 3 rats \pm SEM (student t-test vs. 80 mM glucose: ** $p < 0.1$, *** $p < 0.01$).

Figure 3.2 shows that exposure of the cultured DRG neurons to 80 mM glucose causes a significant increase in the ATF3-level. Furthermore, pre-treatment of DRG neurons with all three growth factors can significantly reduce this neuronal stress, induced by 80 mM glucose. The effect was seen with concentrations between 0.1 ng/ml and 10 ng/ml. Enovin, GDNF and VEGF can bring the ATF3-level back to the level seen in control conditions, with concentrations above 1 ng/ml. Enovin has the largest decrease in the neuronal stress response. There is no effect with concentrations below 0.01 ng/ml. However, there is an increase in the ATF3-expression in DRG-neurons treated with 10 ng/ml GDNF. This might indicate aberrant stimulation by this compound at concentrations of 10 ng/ml or higher.

3.1.3 Time course of neuronal stress in diabetic rats

To document the effect of hyperglycemia on neuronal stress in an ex vivo model, DRG neurons were isolated from control and STZ-treated rats. Only the DRG neurons from the brachial plexus (C7-C8-T1) and the sciatic nerve (L4-L5-L6) were isolated. These nerves innervate the paws of the rat, which correlates with the regions that are the most painful in diabetes mellitus. After one day in culture, neuronal stress was measured by determining the ATF3-level. In this experiment DRG neurons from the diabetic rats were isolated at different time-points after injection of STZ to evaluate the evolution of the neuronal stress over time.

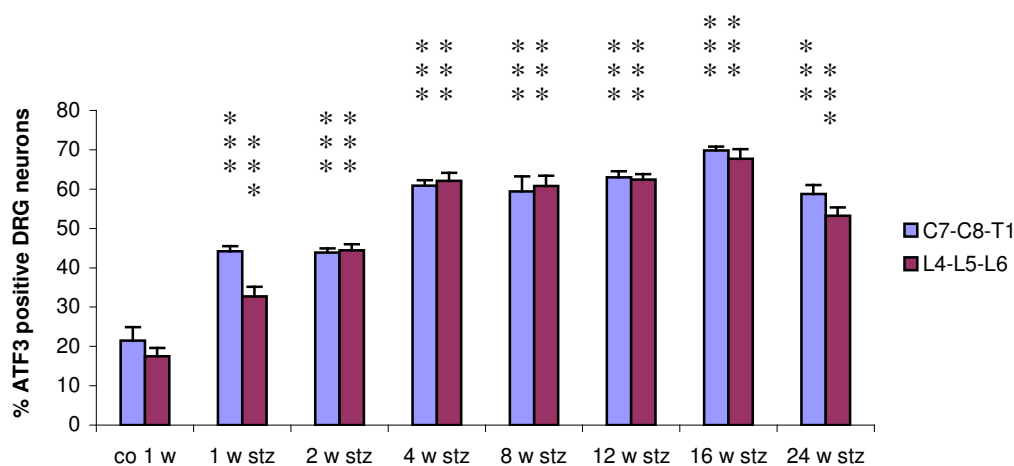


Figure 3.3: Evolution of neuronal stress in diabetic rats. DRG neurons from the brachial plexus and the sciatic nerve were isolated from control- and STZ-treated rats. DRG neurons from the diabetic rats were isolated at different time points after injection of STZ. The cells were plated in 96-well plates and fixed after 24hours. Then they were labeled and the ATF3-level counted. Shown is the mean percentage of 9 wells from 3 rats \pm SEM (student t-test vs. control: *** $p < 0.001$).

A significant higher number of ATF3-positive DRG neurons can be seen in diabetic rats, when compared to the controls. The ATF3-level increases significantly in 1 week and 2 weeks diabetic rats and then reaches a plateau phase with longer episodes of diabetes. This indicates that diabetes mellitus induces neuronal injury in DRG neurons, as measured by the rise in the level of ATF3. The higher vulnerability of the DRG neurons from the diabetic rats to the isolation and culture conditions could also be reflected in the observed increase in the ATF3-level.

3.1.4 Pharmacological modulation of neuronal stress in diabetic rats

To evaluate whether Enovin, GDNF and VEGF also exert a protective effect on diabetes-induced neuronal stress, all DRG neurons were isolated from 8-weeks old diabetic rats. The neurons were cultured in medium containing different concentrations of the growth factors. After one day the neuronal stress response was quantified.

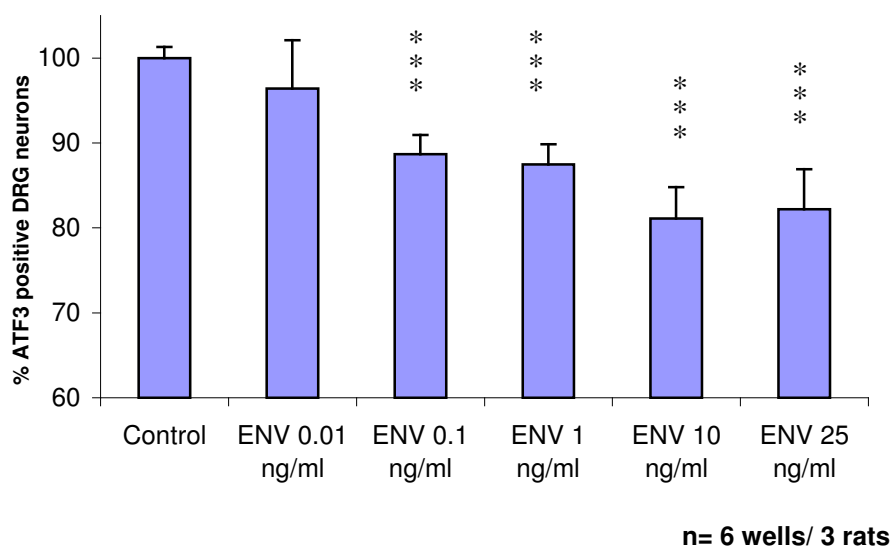


Figure 3.4a: Effect of Enovin on diabetes-induced neuronal stress. DRG neurons were isolated from diabetic rats and plated in 96-well plates containing medium with different concentrations of ENV (0.01 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml and 25 ng/ml). After 24 hours the expression of ATF3 was determined by counting the amount of ATF3-positive neurons in each well. Shown is the mean percentage of 6 wells from 3 rats \pm SEM (student t-test vs. control: *** $p < 0.01$).

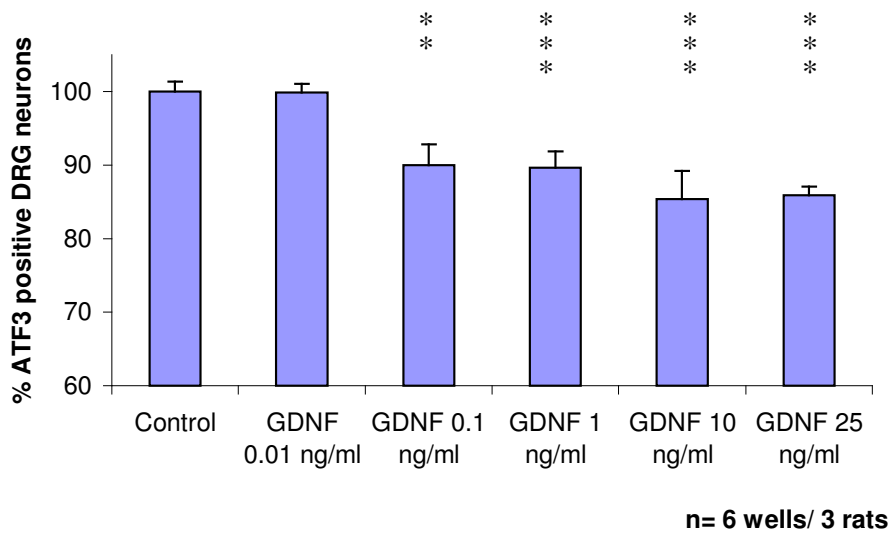


Figure 3.4b: Effect of GDNF on diabetes-induced neuronal stress. DRG neurons were isolated from diabetic rats and plated in 96-well plates containing culture medium with different concentrations of GDNF (0.01 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml and 25 ng/ml). After one day, the percentage of ATF3-positive DRG neurons was determined by counting the amount of ATF3- positive DRG neurons in the samples. Shown is the mean percentage of 6 wells from 3 rats \pm SEM (student t-test vs. control: ** $p < 0.1$, *** $p < 0.01$).

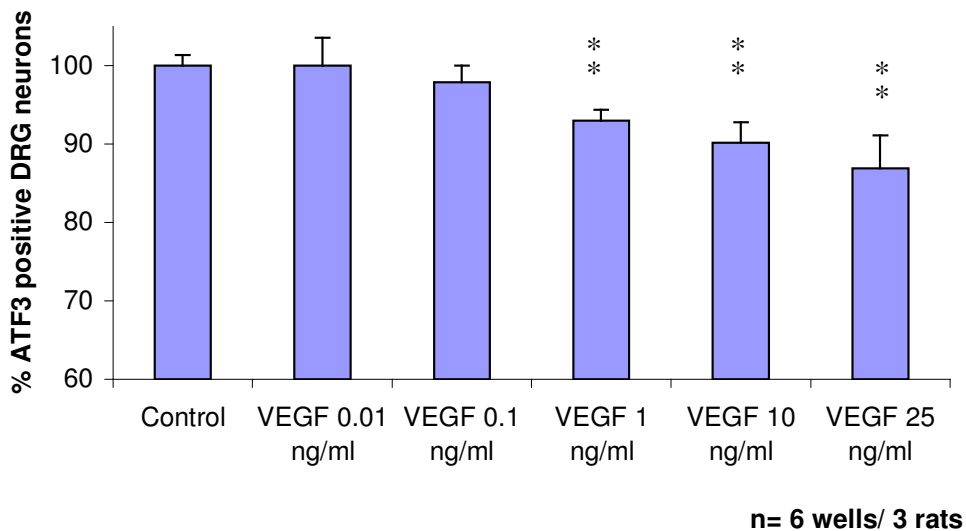


Figure 3.4c: Effect of VEGF on diabetes-induced neuronal stress. DRG neurons were isolated from diabetic rats and plated in 96-well plates containing culture medium with different concentrations of VEGF (0.01 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml and 25 ng/ml). After one day, the percentage of ATF3-positive DRG neurons was determined by counting the amount of ATF3- positive and ATF3-negative DRG neurons in the samples. Shown is the mean percentage of 6 wells from 3 rats \pm SEM (student t-test vs. control: ** $p < 0.1$, *** $p < 0.01$).

This experiment shows that the growth factors can ameliorate neuronal stress, as measured by the ATF3-level, in DRG neurons isolated from diabetic rats. The protective effect of all growth factors was seen with concentrations between 1 ng/ml and 25 ng/ml. Enovin and GDNF decreased the ATF3 level until concentrations of 0.1 ng/ml, whereas VEGF has no effect at this concentration.

Enovin shows an 18% reduction in the neuronal stress response at a concentration of 25 ng/ml. There is a 13% reduction at a concentration of 1 ng/ml. At a concentration of 25 ng/ml, GDNF leads to a 15 % reduction. There is an 11% reduction with this compound at a concentration of 1ng/ml. VEGF reduces the neuronal stress response with 13% with the highest concentration of 25ng/ml, a reduction of 7% was observed at a concentration of 1 ng/ml. According to this numbers, Enovin has the most potent effect on the neuronal stress response.

Taken together, the in vitro experiments, where neuronal stress was induced by a high glucose concentration, and the ex vivo experiment, in DRG neurons from diabetic rats, show that all growth factors have the ability to reduce the neuronal stress response in DRG neurons.

3.2 Neurite outgrowth

In the next part of the study the effect of hyperglycemia on neurite outgrowth was investigated. Therefore DRG neurons, again from the brachial plexus and the sciatic nerve, were isolated at different time points after induction of diabetes. In this way the evolution of neurite outgrowth in cultures from diabetic rats could be evaluated over time.

Furthermore, the effect of growth factors on the neurite outgrowth was investigated.

3.2.1 Evolution of neurite outgrowth in diabetic rats

To measure whether diabetes mellitus also affects neurite outgrowth in an ex vivo model, DRG neurons from the brachial plexus (C7-C8-T1) and the sciatic nerve (L4-L5-L6) were isolated. The DRG neurons were isolated at different time points after injection of STZ to evaluate neurite outgrowth over time. After isolation, the DRG neurons were cultured in 12-well plates and the neurite outgrowth was analyzed after three days.

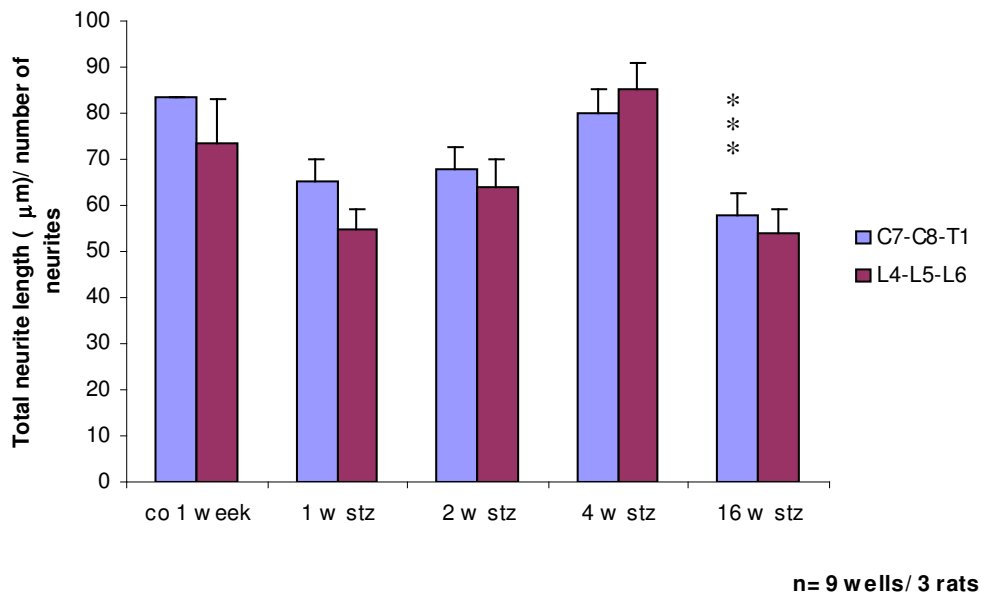
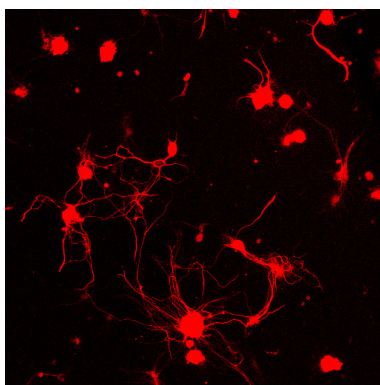


Figure 3.5: Evolution of neurite outgrowth in diabetic rats. DRG neurons were isolated from diabetic rats at different time-points after STZ-injection. The cells were cultured in 12-well plates. After three days neurite outgrowth was quantified by means of image analysis. Shown is the mean of 9 wells from 3 rats \pm SEM (student t-test vs. control: *** $p < 0.001$).

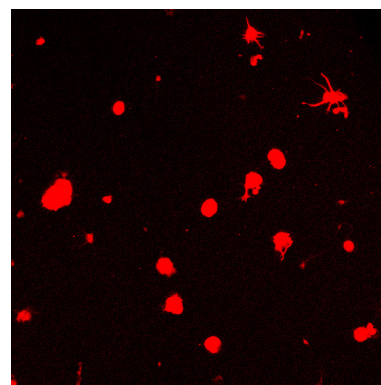
This figure shows that the ratio of total neurite length/ the number of neurites was lower in the diabetic rats compared to the controls. This reduction becomes significant in 16 weeks diabetic rats. However, there is a little elevation at 4 weeks. It might be that the ratio only begins to decrease after 4 weeks diabetes or that the result of the 4 weeks STZ-treated animals are out of the normal growth range, because they are higher than the values of the 2 weeks and 16 weeks diabetic rats. The decrease in the ratio suggests that there is an impaired neurite outgrowth in cultures from diabetic rats.

3.2.2 Pharmacological modulation of neurite outgrowth

The next experiment was performed to investigate the effect of Enovin, GDNF and VEGF on neurite outgrowth. Therefore again all DRG neurons were isolated from control and STZ-treated diabetic rats and cultured in medium containing different concentrations of the growth factors (10 ng/ml, 1 ng/ml and 0.1 ng/ml). In this experiment NGF was used as a positive control, because it has already proven its effect on neurite outgrowth. After three days in culture, neurite outgrowth was analyzed.



A. Control



B. Diabetic

Figure 3.6: Confocal images of neurite outgrowth in DRG neurons. DRG neurons were isolated from control and 16 weeks old diabetic rats. The cells were plated in 12-well plates containing medium with different concentrations of growth factors (10 ng/ml, 1 ng/ml and 0.1 ng/ml). The cells were in culture for three days when they were stained with an antibody against Tau to measure neurite outgrowth. Figure A shows a confocal image of the neurite outgrowth seen in DRG neurons isolated from control rats. Figure B shows a confocal image of the neurite outgrowth seen in DRG neurons isolated from diabetic rats.

The images show that neurite outgrowth was attenuated in DRG neurons obtained from diabetic rats compared to the controls. To quantify this effect, and to document the effect of the three growth factors, the number of cells that display neurite outgrowth were counted.

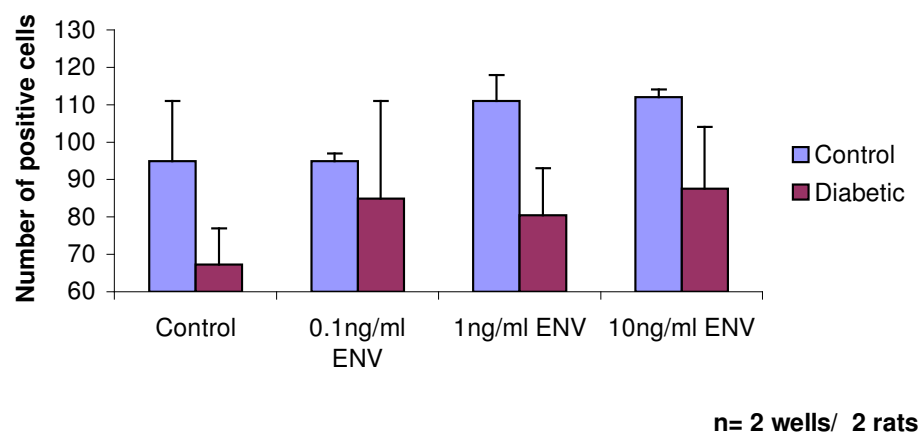


Figure 3.7a: Effect of Enovin on neurite outgrowth. DRG neurons were isolated from control and diabetic rats. The cells were cultured in 12-well plates and treated with different concentrations of Enovin (10 ng/ml, 1 ng/ml and 0.1 ng/ml). After three days neurite outgrowth was quantified by counting the number of cells with neurite outgrowth. Shown is the mean of 2 wells \pm SEM.

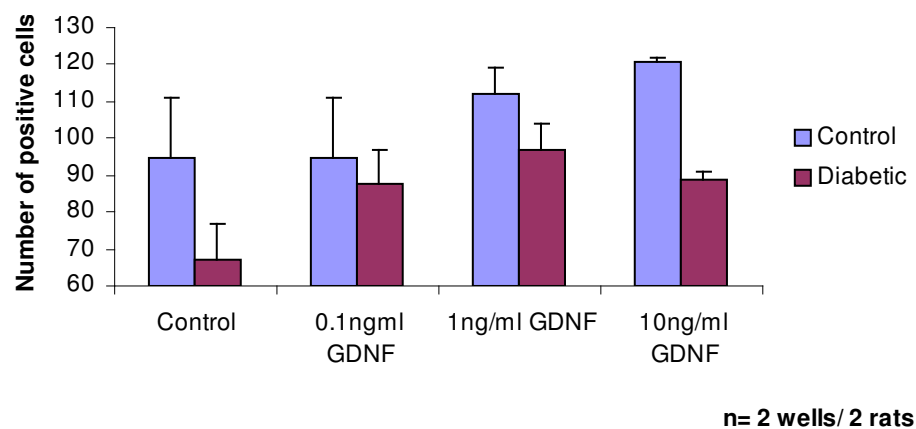


Figure 3.7b: Effect of GDNF on neurite outgrowth. DRG neurons, isolated from control and diabetic rats, were plated in 12-well plates. Different concentrations of GDNF (10 ng/ml, 1 ng/ml and 0.1 ng/ml) were added to the culture medium. After three days neurite outgrowth was quantified by counting the number of positive cells. Shown is the mean of 2 wells \pm SEM.

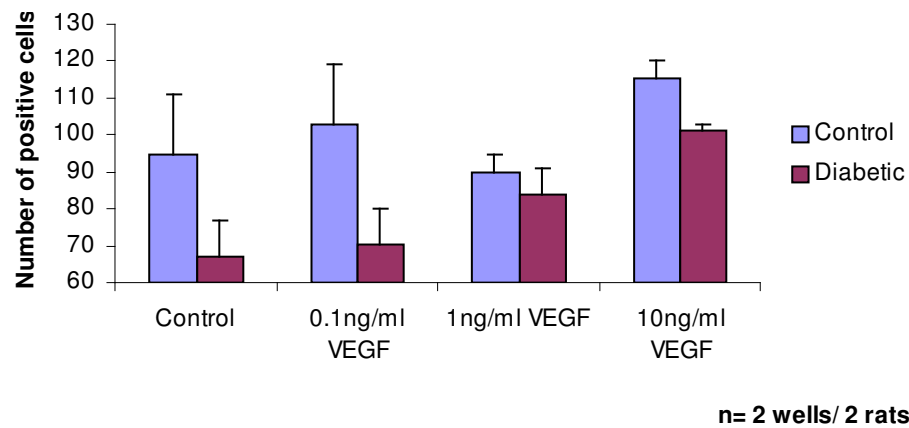


Figure 3.7c: Effect of VEGF on neurite outgrowth. DRG neurons were isolated from control and diabetic rats and plated in 12-well plates containing culture medium with different concentrations of VEGF (10 ng/ml, 1 ng/ml and 0.1 ng/ml). After three days neurite outgrowth was quantified by counting the neurite bearing cells. Shown is the mean of 2 wells \pm SEM.

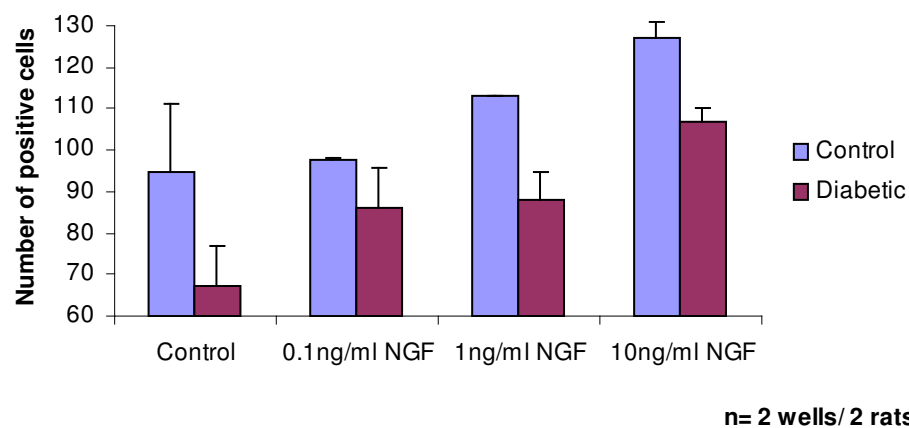


Figure 3.7d: Effect of NGF on neurite outgrowth. DRG neurons were isolated from control and diabetic rats and plated in 12-well plates containing medium with different concentrations of NGF (10 ng/ml, 1 ng/ml and 0.1 ng/ml). Three days after the isolation, neurite outgrowth was quantified by counting the number of positive cells. Shown is the mean of 2 well \pm SEM.

Figure 3.7 shows that the growth factors induce an increase in the number of cells with neurite outgrowth, both in DRG neurons isolated from control- and diabetic rats. Furthermore, the number of neurite bearing cells is lower in the diabetic rats compared to control rats. The increase in the number of positive cells is dose-dependent. The largest increase is observed at a concentration of 10 ng/ml, however the results are not significant. It must be noted that only two wells per condition were measured and that the images were not automatically quantified. The same experiment must be repeated several times and image analysis must be applied to obtain complete data.

3.3 Neuronal viability

In the last part of the study, the viability of dorsal root ganglion (DRG) neurons after the isolation procedure was investigated. To see whether the increased neuronal stress seen in diabetic rats also reflects an increase in cell death, neuronal viability in cultures of diabetic rats was examined.

3.3.1 Evolution of neuronal viability in diabetic rats

To investigate whether diabetes mellitus induces a decrease in neuron viability, DRG neurons were isolated at different time points after STZ injection. Only DRG neurons from the brachial plexus and the sciatic nerve were used in the experiment. After three days in culture, the viability of the neurons was determined by counting the apoptotic cells in the cultures.

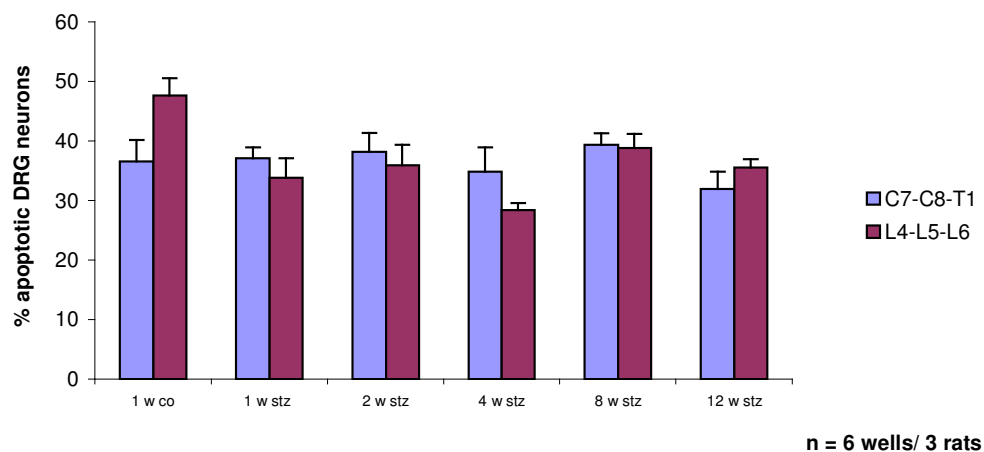


Figure 3.8: Evolution of neuronal viability in diabetic rats. DRG neurons were isolated from diabetic rats at different time points after induction of the diabetic episode. The cells were cultured in 12-well plates. After three days neuronal viability was documented. Shown is the mean number of viable neurons counted in 6 wells from 3 rats \pm SEM.

This figure shows that the number of apoptotic DRG neurons in diabetic rats is not significantly different compared to control rats. The number of apoptotic DRG neurons did not change over time, from 1 week to 16 weeks after STZ-injection.

4. Discussion

In this study the pathogenesis of diabetic neuropathy was investigated. It was already shown that high-glucose levels and an insufficient neurotrophic support are important factors in the onset of diabetic neuropathy. Therefore the effect of glucose on rat dorsal root ganglion (DRG) neurons was investigated in both an *in vitro* and *ex vivo* models for diabetic neuropathy. The beneficial effects of different growth factors in these models were documented.

The first part of the study investigated the effect of hyperglycemia on DRG neurons in both an *in vitro* and *ex vivo* model for diabetic neuropathy. The results from both models indicate clear differences between neurons under control- and hyperglycemic conditions. In both models an increase in the neuronal stress response was observed. *In vitro*, where DRG neurons were exposed to different concentrations of glucose, an increased neuronal stress response was observed, as indicated by the ATF3 level. The effect was seen with concentrations between 10 mM and 50 mM of glucose. Addition of 80 mM glucose further elevates the ATF3-level. At this concentration the ATF3-level has almost doubled. The *ex vivo* model, where DRG cultures of diabetic rats were used, also showed an increase in the neuronal stress response. The rise is already apparent in 1-week diabetic rats; a plateau phase is reached in diabetic rats that were longer than 4-weeks diabetic. In the *ex vivo* model we also investigated the effect of the diabetic episode on neurite outgrowth and neuronal viability. A decrease in the average length per neurite was observed indicative for impaired neurite outgrowth in cultures from diabetic rats. There was no significant difference in the number of apoptotic cells compared to the controls.

In the other part of the study the effects of different growth factors (ENV, GDNF and VEGF) on hyperglycemia-induced changes in DRG neurons were evaluated. Again the neuronal stress response, neurite outgrowth and neuronal viability were used to quantify the effect. *In vitro* and *ex vivo*, all tested growth factors were able to reduce the hyperglycemia-induced neuronal stress. Furthermore, when DRG neurons from control and diabetic rats were treated with different concentrations of Enovin, GDNF and VEGF an increase in the number of neurite expressing cells was observed.

NGF was used as a positive control in this experiment because it has proven its effect on neurite outgrowth. These findings indicate the potential for the disease modifying properties of Enovin, GDNF and VEGF in conditions of peripheral diabetic neuropathy.

4.1 Effect of hyperglycemia on DRG neurons

In the first part of the study, the effect of hyperglycemia on DRG neurons was investigated in both an in vitro and ex vivo model for diabetic neuropathy. In vitro, DRG neurons isolated from control rats were exposed to different concentrations of glucose in the culture medium. DRG neurons isolated from diabetic rats, at different time points after STZ-injection, were used as an ex vivo model for diabetic neuropathy. To study the effect of high-glucose levels on the DRG neurons, the neuronal stress response, neurite outgrowth and neuronal viability were measured.

In this study the expression of ATF3 was used as a marker for neuronal stress. Almost all reports used immunocytochemistry to label for ATF3. Previous studies showed that there is a clear correlation between nerve damage and the level of ATF3 expression. Tsujino et al. (2000) were among the first to report the use of ATF3 as a neuronal marker for cellular stress. They induced neuronal injury by transection of the sciatic nerve in rats, which resulted in an induction of ATF3 expression in virtually all DRG neurons that were axotomized (36).

In the study of Shortland et al. (2006) it was investigated whether a lesion of the L5 spinal nerve also induces ATF3-expression in adjacent neurons. Their results suggest that ligation of L5 induced expression of ATF3 in the L5-nerve but also in the adjacent L4 region. This indicates that the ATF3-expression in the L4 DRG neurons results from unintentional damage caused by the surgical procedure. Therefore caution is warranted when interpreting the phenotypic plasticity of DRG neurons in adjacent ganglia (42). Obata et al. (2002) used a chronic constriction model of the sciatic nerve, consisting of both intact and injured peripheral axons. They also reported an increased number of ATF3-positive DRG neurons in the L4-L5 region (43). All these reports indicated ATF3 as a marker for neuronal injury. However, most studies used a chronic constriction models. Only a few articles investigated the effect of hyperglycemia on ATF3-expression. Wright et al. (2004) used an experimental mouse model of diabetic neuropathy to study the expression of ATF3 by sensory neurons.

ATF3-expression was already apparent 3 weeks after injection of STZ to induce diabetes. This result demonstrates that the diabetes-induced damage of sensory neurons can induce the expression of genes like ATF3 linked to peripheral nerve injury (35).

In this research an increased expression of ATF3 in both an *in vitro* and *ex vivo* model for diabetic neuropathy was observed, confirming the results of Wright et al in mice. This indicates that hyperglycemic conditions induce damage in DRG neurons from rodents, which contributes to the development of diabetic peripheral neuropathy. As such ATF3 could be used as a biomarker for neuronal damage in diabetic conditions.

The second parameter that was used to study the effect of hyperglycemia on DRG neurons was neurite outgrowth. Only an *ex vivo* model was used. Previous work of Russell et al. (1999) already reported that neurite outgrowth is dependent on the concentration of glucose in the medium. In cultured embryonic DRG neurons an optimal neurite outgrowth was observed with 30 mM glucose in the culture medium. However, the outgrowth was significantly reduced with concentrations of 20 mM or 40 mM glucose in the medium of the cells. This indicates that only a narrow range of glucose produces optimal neurite outgrowth (44).

In the current study neurite outgrowth was evaluated at different time points after the induction of diabetes. The results showed a decrease in neurite outgrowth in DRG neurons from diabetic rats compared to the controls. Furthermore, this effect is more apparent in the rats that are diabetic for a longer period. This indicates that neurite outgrowth is impaired in cultures from diabetic rats. The longer episodes of diabetes make it harder for DRG neurons to overcome the hyperglycemia-induced damage.

The last argument for glucose-induced neuronal damage is the theory that hyperglycemia activates caspase pathways that ultimately leads to apoptosis. The latter could be a contributing factor in the pathogenesis of diabetic peripheral neuropathy. The group of Russell et al. (1999) investigated the effect of hyperglycemia on neuronal viability. They reported that glucose induces a dose-dependent effect on caspase-cleavage. There was an optimal survival of DRG neurons when *in vitro* a concentration of 25 to 30 mM glucose was added to the medium of the neurons. The relative high concentration of glucose most likely reflects the increased energy requirements and metabolic rate of DRG neurons. Higher or lower concentrations of glucose decreased the neuronal viability of the cells (45).

Srinivasan et al. (2000) used an in vivo model from control and 3- to 6-weeks old diabetic rats. They used the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method to detect apoptotic changes. The results of this study show an increased number of apoptotic neurons in DRG neurons from diabetic rats when compared to control rats (46). Schmeichel et al (2003) evaluated the effects of chronic hyperglycemia on DRG neurons using immunocytochemistry and electrophysiological techniques to detect oxidative injury in diabetic rats at different time points after STZ-injection. They reported electrophysiological abnormalities and an increase in caspase-3 labeling at 1, 3 and 12 months after STZ-injection. Furthermore, according to this group the percentage of apoptotic neurons increased according to the length of the duration of the diabetic episode (47).

The results of the present study show no significant changes in the number of surviving DRG neurons in cultures of diabetic rats compared to those from control rats. An explanation for this could be that the apoptotic cells are lost during the isolation or culturing procedure.

4.2 Effect of neurotrophic support on DRG neurons

Deficient neurotrophic support has been proposed to contribute to the development of diabetic neuropathy. In the current study the potential beneficial effects of growth factors on DRG neurons were investigated by measuring the ATF3-level, neurite outgrowth and neuronal viability.

Previous studies already investigated the beneficial actions of growth factor treatment on diabetes-induced neuronal damage. Akkina et al. (2001) demonstrated that diabetes mellitus induced damage to sensory neurons and that GDNF administration to STZ-treated mice can selectively reverse these effects (48). Similar results have been reported by Averill et al. (2004). This group transected the sciatic nerve in rats. Their data show that ATF3 expression can be modulated by supplementation of NGF and GDNF and suggests that ATF3 expression may be induced by the loss of target-derived NGF and GDNF (49). Christianson et al. (2003) evaluated whether administration of NGF, GDNF or NRTN to diabetic rats for two weeks could induce axonal branching. The data indicate that treatment with GDNF or NRTN strongly stimulated axon growth and branching (50).

Evidence has emerged that the vascular growth factor VEGF also has direct effects on neurons and glial cells and stimulates their growth and survival. Veves et al. (2001) reported that VEGF gene transfer 12 weeks after the induction of diabetes in rats fully restored nerve function abnormalities in both large and small fibers (31). Samii et al. (1999) used a monoclonal antibody to VEGF to stain its expression in the sciatic nerve and DRG neurons from STZ-treated rats. Diabetic rats with sensory neuropathy expressed VEGF in peripheral nerves and spinal ganglia. Age-matched animals did not express this protein. In addition, improvement of functional state of neuropathic nerves and ganglia, as achieved by application of insulin, decreased VEGF-immunoreactivity in these tissues (51). These results indicate that VEGF may play a role in complete nerve regeneration and its regulation may reflect the functional state of the peripheral nerves.

The current study shows that Enovin, GDNF and VEGF can all protect the DRG neurons against glucose-induced damage measured by the ATF3-level and neurite outgrowth. All different growth factors decreased neuronal stress in the in vitro and ex vivo model. The addition of these compounds to the culture medium of DRG neurons, isolated from control- and STZ-treated rats, also increased the number of neurite expressing cells. However, the results were not significant. More experiments must be carried out to draw reliable conclusions.

Taken together these results indicate a beneficial effect of all used growth factors on diabetes mellitus-associated neuronal damage. These substances are therefore possible candidates for the treatment of diabetic peripheral neuropathy.

4.3 General conclusion

The data of this study together with the results of other research gives a better insight in the development of diabetic peripheral neuropathy. It has become clear that hyperglycemic conditions contribute to the pathogenesis of diabetic peripheral neuropathy. In addition, it was documented that growth factors could protect the DRG neurons against this hyperglycemia-induced damage. Both the in vitro and ex vivo model showed that hyperglycemic conditions leads to an increased neuronal stress response and a decreased neurite outgrowth.

Addition of different growth factors can reduce the neuronal stress, as measured by the ATF3-level, and leads to a rise in neurite outgrowth.

Increasing evidence indicates that the increased production of reactive oxygen species (ROS) is a key player in the onset of diabetic peripheral neuropathy. The mitochondria are very susceptible to oxidative damage. The damage caused by ROS not only leads to neuronal stress but also a depletion of the ATP-levels, which is disastrous for the energy-demanding neurons. Neurite outgrowth, which is an energy-consuming process, is therefore disabled in hyperglycemic conditions. Crucial in the pathogenesis of diabetic neuropathies is the release of cytochrome c from the outer mitochondrial membrane. Cytochrome c can activate caspase-3. The net effect is apoptosis of the DRG neurons (47)

The second factor, which is important in the pathogenesis of diabetic peripheral neuropathy, is a deficient neurotrophic support. The neuroprotective factors that were used in this study ultimately activate the PI3K pathway. This pathway has several protective effects like upregulation of hexokinase and NADH, which leads to an increase in ATP-production. There is also an increased production of the anti-apoptotic Bcl-2. This protein stabilizes the mitochondrial membrane by inhibiting the opening of a large conductance channel, called the mitochondrial permeability transition pore (PT). Opening of the PT will ultimately lead to rupture of the outer membrane and therefore release of cytochrome c. Bcl-2 also protects the cell after cytochrome c release. Bcl-2 can prevent apoptosis by enhancing proton extrusion from mitochondria that help to maintain the mitochondria buffering capacity (46). These beneficial effects are lost without the support of the growth factors.

The data in this study shows the potential of Enovin, GDNF and VEGF as possible candidates for the treatment of diabetic peripheral neuropathy. In vitro and ex vivo studies indicate that these factors can reduce the glucose-induced neuronal stress and stimulate neurite outgrowth. These results are very promising and warrant further research to confirm the disease modifying potential of these compounds. In vivo testing, for example, must further confirm the positive results obtained in this research.

Nevertheless these promising results provide us new perspectives in finding therapeutic treatments for diabetic peripheral neuropathy.

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