The role of growth factors in diabetic peripheral neuropathy

Gina M. Leinninger,[1] Andrea M. Vincent,[2] and Eva L. Feldman[1,2]

Neuroscience Program[1] and Department of Neurology,[2] University of Michigan, Ann Arbor, MI, USA

Abstract Peripheral neuropathy afflicts 60% of all diabetic patients. Underlying the clinical disorder is the loss or degeneration of neurons, Schwann cells, and neuronal fibers. This degenerative pathology has prompted interest in the potential of growth factors as a therapy in diabetic neuropathy. Three lines of evidence support the theory that growth factors may be important in this disorder: (1) endogenous growth factors promote survival and health of neurons, (2) expression levels of growth factors are altered in diabetic neuropathy and peripheral neuron injury, and (3) growth factors induce neuronal regeneration in *in vitro* and *in vivo* models of diabetic injury. This review surveys the roles of several growth factors in diabetic neuropathy, including the neurotrophins, insulin-like growth factors, cytokine-like growth factors, and vascular endothelial growth factor. These growth factors are examined in terms of their expression during peripheral nerve injury and their protective and regenerative effects on peripheral neurons. Growth factor-mediated neuroprotective signaling is discussed, particularly in relation to the recent research, suggesting that diabetic neuropathy-induced degeneration stems from oxidative stress. Finally, the potential of growth factors as therapeutic agents is addressed, including an assessment of past growth factor clinical trials and other potential avenues of growth factor therapy.

Key words: diabetic neuropathy, growth factor, insulin-like growth factor-I, nerve growth factor, oxidative stress

Diabetic Peripheral Neuropathy

Neuropathy is the most common complication of diabetes, occurring in 60% of all diabetic patients *(Feldman et al., 1999)*. Although the pathogenesis of neuropathy itself is unknown, results of the diabetes control and complications trial (DCCT) link neuropathy to hyperglycemia *(The Diabetes Control and Complications Trial Research Group, 1993)*. The development of neuropathy is a late event in diabetes mellitus, often not manifesting until 20 or more years after disease onset. Diabetic neuropathies can be difficult to diagnose. Primarily, any alternative cause of neuropathy must be ruled out, such as those secondary to drug treatment, other metabolic disease, or trauma. In addition, multivariate symptoms can occur including pain, wasting and weakness of lower limb muscles (proximal motor neuropathy), or erectile and bladder dysfunction (autonomic neuropathy). The most common peripheral neuropathies are sensory, in which symptoms in the limbs can be either positive (pain, excessive sensitivity to temperature) or negative (numbness, loss of sensory perception) *(Boulton and Malik, 1998)*. Sensory peripheral neuropathy most often affects the feet and legs, although with disease progression, the hands and arms may also be affected. Loss of sensation in the feet is the most common effect of diabetic neuropathy and promotes significant risk to the limb. Due to decreased tactile and pain perception, injuries to the feet are often undetected by the patient and thus may go unchecked and untreated. Successive

Address correspondence to: Eva L. Feldman, MD, PhD, Department of Neurology, University of Michigan, 4414 Kresge III, 200 Zina Pitcher Place, Ann Arbor, MI 48109, USA. Tel: +1-734-763-7274; Fax: +1-734-763-7275; E-mail: efeldman@umich.edu

injuries manifest as non-healing foot ulcerations, and progressive damage to the feet can ultimately result in severe infection, gangrene, and amputation *(Vileikyte, 2001)*. This dire sequence of events accounts for 60% of all lower extremity amputations in the general population *(Feldman et al., 1998)*. Thus, peripheral neuropathy presents a serious toll on patient well being and health care costs. However, despite the devastating effects and widespread occurrence of diabetic peripheral neuropathy, there is no treatment outside overall control of the diabetic condition itself *(Vinik et al., 2000)*.

The past 20 years of research have investigated the pathogenesis of diabetic peripheral neuropathy, seeking to elucidate points for therapeutic intervention. Although the pathogenetic mechanism is not fully understood, the disorder is pathologically marked by degeneration of Schwann cells and myelinated neuronal fibers as well as loss of a population of the neurons located in the dorsal root ganglia (DRG) *(Chopra et al., 1977; Sendtner et al., 1991; Greene et al., 1992; Kalichman et al., 1998; Schmeichel et al., 2003)*. These degenerative markers led researchers to investigate growth factors as potential therapeutic agents in the treatment of diabetic neuropathy, as supported by two early lines of research: (1) endogenous growth factors promote survival and health of neurons and (2) growth factors induce neuronal regeneration in *in vitro* and *in vivo* models of neurological injury and disease. These findings support the hypothesis that growth factors may be beneficial in preventing neuronal damage or regenerating already damaged neurons in patients with diabetic neuropathy. In particular, the neurotrophins, the insulin-like growth factors (IGFs), the cytokine-like growth factors, and vascular endothelial growth factor (VEGF) have promise as therapeutics. This review will address the roles of these growth factors in normal systems, how growth factor levels and functions are altered in diabetes and the evidence for utilizing growth factors as therapeutics for diabetic peripheral neuropathy.

Growth Factors of the Peripheral Nervous System

The neurotrophins

The neurotrophins are a family of factors that regulate the growth and survival of the central and peripheral nervous systems. Neurotrophins provide two forms of support that are critical to the development and maintenance of the nervous system: (1) trophic support (by promoting survival and/or growth of neurons) and (2) tropic support (by directing the movement of extending neurites). The trophic and tropic support of these factors promotes the birth and maintenance of healthy and appropriately connected neurons. Thus, neurotrophins are vital for nervous system development and function.

The classic neurotrophin family is comprised of five molecules: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins-3 (NT-3), -4/5 (NT-4/5), and -6 (NT-6) *(McDonald and Murray-Rust, 1995)*. These molecules share about 50% sequence homology, suggesting their potent evolutionary importance in nervous system regulation *(Lindsay et al., 1994)*. Although they are similar in sequence, individual neurotrophins mediate their particular actions by binding to distinct cell types, as determined by the cellular expression of their corresponding receptors. The neurotrophin receptors, like their cognate ligands, are also similar in sequence. These receptors are collectively referred to as tropomyosin-related kinases (Trks), originally named because the first isolated Trk protein was a chimera of Trk and non-muscle tropomyosin from a colon carcinoma biopsy. Functional Trk receptors contain a conserved tyrosine kinase-signaling domain and hence are also collectively referred to as receptor tyrosine kinases. Trk receptors are oriented in cell membranes, such that their ligand-binding sites are extracellular and their tyrosine kinase domains lie in the internal cytoplasm *(Hardie, 1995)*. Trk A is the high-affinity receptor for NGF, Trk B is the receptor for BDNF and NT-4/5, whereas Trk C primarily serves as the receptor for NT-3 *(Barbacid, 1994)*. Binding of a cognate ligand to its receptor stimulates intracellular receptor tyrosine kinase phosphorylation, which then activates intracellular signaling. Thus, the tyrosine kinase domain of the Trk receptors translates the extracellular growth factor message to the neuron, where this message can be interpreted and functionally enacted *(Hardie, 1995)*. There is also a low-affinity neurotrophin receptor, known as p75, that is unrelated to the Trk receptor family. The p75 receptor can bind all members of the neurotrophin family. This indiscriminate, low-affinity binding can direct the neurotrophins to their high-affinity Trk receptors to promote neurotrophic intracellular signaling *(Friedman and Greene, 1999)*. However, in the absence of Trk receptors, p75 has been implicated in promoting apoptosis in response to levels of unprocessed pro-NGF *(Ibanez, 2002)*.

By far, NGF is the best understood of all the neurotrophin family. NGF was initially reported by Rita Levi-Montalcini, describing the metabolic effects of NGF on sensory and sympathetic chick ganglia *(Liuzzi et al., 1965)*. Since then, NGF has been shown to promote survival and neuritogenesis in primary neurons of the central and peripheral nervous systems *(Sofroniew et al., 2001)* as well as in many neuron-like

cell lines *(Levi et al., 1988)*. The importance of NGF in the peripheral nervous system is well illustrated by its developmental expression pattern. NGF is synthesized by target organs of sympathetic and neural-crest-derived DRG sensory neurons and by Schwann cells ensheathing the fibers to these targets *(Bandtlow et al., 1987)*. Trk A receptors are also highly expressed in DRG during peripheral nervous system development *(Yan and Johnson, 1987)*. Removing NGF signaling is devastating to the developing nervous system. This is demonstrated by NGF homozygous knockout mice, which do not develop proper sympathetic neurons or small neural crest-derived sensory neurons *(Crowley et al., 1994)*. Similarly, Trk A knockout mice exhibit decreased survival of sympathetic neurons after embryonic day 15.5 and compromised innervation of distal targets *(Fagan et al., 1996)*. Thus, the NGF system is critical to peripheral nervous system development and preservation.

Comparatively less is known about the importance of the other neurotrophins in the peripheral nervous system. BDNF supports sensory neurons derived from both the neural crest (DRG) and the placode (nodose ganglion), whereas NGF supports only cells derived from the neural crest. BDNF may also support motor neuron survival and development, indicating that it has a broader trophic range compared to NGF *(Schecterson and Bothwell, 1992; Sendtner et al., 1992)*. The trophic role of NT-3 includes support of neurons from the neural crest and the placode as well as neurons of the paravertebral chain sympathetic ganglia *(Maisonpierre et al., 1990; Henion et al., 1995)*. NT-4/5 is expressed in facial motor neurons and protects these from injury-induced death *(Koliatsos et al., 1994)*. Similar to NGF, knockout mice have also revealed much about the developmental roles of the other neurotrophins. For example, combined BDNF and NT-4/5 knockout mice lack neural placode-derived sensory cranial ganglia, whereas NT-3 knockouts lack type Ia sensory afferents and muscle spindles, demonstrating the need for neurotrophic support during development *(Snider, 1994; Conover et al., 1995)*. In summary, the neurotrophins are critical to formation and function of the peripheral nervous system and loss of neurotrophin signaling in development or due to injury results in devastating effects. The importance of these factors in normal nervous system development and maintenance also points to them as promising factors in regeneration of peripheral neurons lost or damaged due to disease states.

Insulin-like growth factors

IGF-I and IGF-II are neurotrophic factors with sequence homology to pro-insulin. IGFs mediate their neurotrophic effects via the type I IGF receptor (IGF-IR), which, like the Trk receptors, is a receptor tyrosine kinase *(Daughaday and Rotwein, 1989; Sara and Hall, 1990)*. IGFs are especially important in the nervous system, where they are vital in establishing nervous system architecture. IGFs are required for normal fetal and post-natal development of humans and rats *(Sara et al., 1981; Rotwein et al., 1987)*. IGF-II is a major factor in central nervous system development, because it is expressed in the brain, vascular structures of the nervous system, and in motor neurons *(Haselbacher et al., 1985; Hansson et al., 1988; Couce et al., 1992; Logan et al., 1994; Hammarberg et al., 1998)*. IGF-I is more broadly distributed throughout the nervous systems and is normally expressed in craniofacial sensory ganglia *(Ayer-LeLievre et al., 1991)*, sciatic nerve, spinal cord, sensory DRG, and brain *(Rotwein et al., 1988; Bondy et al., 1990; Devaskar et al., 1993; Syroid et al., 1999; Craner et al., 2002)*. The IGF-IR is also widely expressed throughout the peripheral nervous system, including sensory DRG *(Daughaday and Rotwein, 1989)* and Schwann cells of peripheral nerves *(Cheng et al., 1996b)*. The broad expression of both IGFs and their cognate receptor in the peripheral nervous system suggests the importance of the IGF system in normal development and nervous system maintenance. The IGF system also has a role in the damaged peripheral nervous system. IGF-I promotes nerve repair following injury, suggesting that it may be useful in treating nerve damage due to trauma or neurodegenerative disease *(Gehrmann et al., 1994; Pu et al., 1995; Cheng et al., 1996a)*.

Like NGF, IGF-I mediates neurotrophic effects in many different neurons of the peripheral nervous system, including peripheral sensory *(Ishii et al., 1994)*, sympathetic *(Ishii et al., 1994; Zackenfels et al., 1995)*, and motor neurons *(Cheng et al., 1996a; Vergani et al., 1998; Pu et al., 1999)*. The neurotrophic roles of IGFs in the peripheral nervous system are particularly important, because the IGFs are the only growth factors found in nerve and muscle that can support both sensory and motor nerve regeneration in adult animals *(Glazner et al., 1993; Ishii and Marsh, 1993; Lewis et al., 1993; Houenou et al., 1994; Contreras et al., 1995; Ishii, 1995)*. In addition to survival and differentiation, IGFs also promote neurite formation and outgrowth in sensory, sympathetic, and motor neurons of the peripheral nervous system *(Prager and Melmed, 1993; Vergani et al., 1998; Russell and Feldman, 1999)*. Effects of IGF-I are not confined to neurons, because IGF-I also stimulates Schwann cell mitogenesis and neuronal fiber myelination *(Cheng et al., 1996a; Sondell et al., 1997; Cheng et al., 1999; Ye et al., 2002)*. These Schwann cell-mediated effects are critical to appropriate inter-neuronal signaling and peripheral nervous system function.

The importance of IGF-I in promoting the health and connectivity of the peripheral nervous system is confirmed by the effect of IGF-I knockout in mice. IGF-I knockouts display decreased motor and sensory nerve conduction velocities, and their peripheral neurons have smaller axonal diameters *(Gao et al., 1999)*. Knockout of the IGF-IR is embryonic lethal, demonstrating how vital the IGF-I-signaling system is to development. Combining the evidence of the peripheral nervous system expression pattern of IGF and the profound effects of the IGF system on neuronal architecture, maintenance, and regeneration, points to IGF-I as a potential therapeutic agent in the treatment of peripheral nervous system disease.

Cytokine-like growth factors

A third family of trophic factors that are important in the peripheral nervous system are the cytokine-like growth factors. Of this family, ciliary neurotrophic factor (CNTF) and glial-derived neurotrophic factor (GDNF) are capable of supporting peripheral neurons.

CNTF was first documented as a factor that caused cultured chick paravertebral sympathetic ganglia, which are normally proliferative, to enter a program of neuronal differentiation *(Ernsberger et al., 1989)*. Since its initial discovery, CNTF has been repeatedly shown to have trophic actions in the nervous system *(Kuzis and Eckenstein, 1996)*. CNTF expression in the peripheral nervous system is mainly limited to sciatic nerve *(Lin et al., 1989)*, Schwann cells, and astrocytes *(Friedman et al., 1992; Lee et al., 1995)*. The levels of CNTF vary during development, being low during embryonic stages but higher in the adult. This suggests that CNTF does not function as a target-derived growth factor in development. Instead CNTF may be required to maintain and repair mature neurons. In the adult animal, CNTF proteins are not secreted but are instead retrogradely transported by sensory and motor neurons following injury *(Sendtner et al., 1990; Curtis et al., 1993)*. Although CNTF lacks the target-derived dispersal mechanism of the classical neurotrophins, trophic support by CNTF is well documented in cultured motor neurons, whereas NGF, BDNF, and NT-3 cannot support these neurons *(Sendtner et al., 1991)*. Also, *in vivo*, CNTF mainly supports motor neurons, including those of injured facial and sciatic nerves *(Li et al., 1994; Sendtner et al., 1997)*. The trophic support of CNTF is modulated through binding to the CNTF receptor. Unlike the classical Trk and IGF-I receptors, the CNTF receptor is not a heterodimeric receptor tyrosine kinase. Rather, the CNTF receptor is actually composed of three components: (1) CNTF receptor α (CNTFRα), which determines ligand specificity, (2) gp130, and (3) leukemia inhibitory factor receptor β (LIFRβ). Together, both gp130 and LIFRβ are referred to as the β components *(Richardson, 1994)*. The expression of CNTFRα, which is responsible for CNTF binding, is mainly limited to the nervous system, including brain, perikarya, and neuronal processes of adult spinal cord, DRG, and peripheral nerve *(MacLennan et al., 1996)*. The β components are expressed more widely and are also components of receptors for other cytokines. Thus, expression of CNTFRα alone determines whether a cell can respond to CNTF and mount a trophic response. Mice with gene deletions of the CNTF system provide further evidence that this system may be more important in repair than in development. For example, mice with targeted disruptions in the CNTF genes only develop mild degenerating motor neuron symptoms, which appear slowly over time. Developmental expression of leukemia inhibitory factor (LIF) in these mice compensates for the lack of CNTF, indicating that CNTF is not itself vital for proper motor neuron development *(Sendtner et al., 1996)*. However, mice lacking CNTF that underwent facial nerve lesioning or that were subjected to myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (a model of multiple sclerosis) exhibited larger reductions in motor neuron survival compared to respective control transgenic mice *(Sendtner et al., 1997; Linker et al., 2002)*. Therefore, these knockout models clearly implicate CNTF as an important factor in nervous system injury.

GDNF is a recently described trophic factor, originally isolated in 1993 from a rat glial-derived cell line *(Lin et al., 1993)*. A member of the transforming growth factor-β family, GDNF was first cited as a trophic support factor for dopaminergic neurons of the central nervous system. However, further work has demonstrated the trophic actions of GDNF on autonomic, sensory, and motor neurons of the peripheral nervous system *(Buj-Bello et al., 1995; Oppenheim et al., 1995; Baumgartner and Shine, 1998)*. GDNF is expressed in the developing spinal cord and in target tissue of motor neurons *(Oppenheim et al., 1995; Nosrat et al., 1996)*. The GDNF receptor, through which GDNF exerts its neurotrophic ligand signaling, is composed of the glycosylphosphatidylinositol (GPI)-linked α subunit (GDNFRα) and a transmembrane tyrosine kinase protein, Ret *(Durbec et al., 1996; Treanor et al., 1996)*. Both GDNF receptor components are expressed throughout the peripheral nervous system. In the mouse, Ret is widely expressed during development, including expression within spinal cord motor neurons, whereas the GDNFRα is expressed in both nerve and spinal cord. Both components are also present in most cranial motor nuclei *(Naveilhan et al., 1997; Mikaels et al., 2000)*. Like the role of the CNTFRα receptor component, the GDNFRα receptor component is required for GDNF signaling, whereas

the Ret portion of the receptor that is comparable to the CNTF receptor β components has functions outside GDNF signaling. Thus, GDNFRα-deficient mice have reduced response to GDNF and subsequently fail to form an enteric nervous system *(Tomac et al., 2000)*. GDNF knockouts also exhibit severe peripheral nervous system deficits, including reductions in spinal and cranial motor neurons, whereas the survival of spinal sensory neurons in DRG is unaffected *(Oppenheim et al., 2000)*. Hence, GDNF, like CNTF, appears to be mainly neurotrophic for motor neurons and not sensory neurons of the peripheral nervous system. However, although peripheral nerve injury upregulates GDNF in motor neurons, GDNF is also upregulated in Schwann cells proximal and distal to a sciatic nerve crush site *(Hammarberg et al., 1996; Hottinger et al., 2000)*. Thus, GDNF holds promise as a potential therapeutic agent in the treatment of both motor and sensory neuron damage, secondary to peripheral nervous system disease.

Vascular endothelial growth factor

VEGF has recently been the subject of interest due to emerging results about its effects on neurons and glia. Discovered in 1983, VEGF was first studied as a promoter of angiogenesis *(Ferrara, 2001)*. However, further study has implicated VEGF as a potential therapeutic in such nervous system disorders as amyotrophic lateral sclerosis (ALS), Alzheimer's Disease, stroke, and peripheral neuropathy *(Carmeliet and Storkebaum, 2002)*. There are two receptors for VEGF: VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). Both are receptor tyrosine kinases. Although VEGFR-2 transduces intracellular signals in response to VEGF binding, VEGFR-1 receptors do not seem to function in signal transduction but rather act to modulate free VEGF levels *(Murphy et al., 1991)*. Although there is little data about normal VEGF expression during peripheral nervous system development, it is clear that VEGF treatment promotes neurotrophic effects in cells of the peripheral nervous system. For example, VEGF induces Schwann cell proliferation, stimulates axonal outgrowth of neurons, and promotes increased survival in both the neurons and Schwann cells of superior cervical ganglia and DRG. These responses are blocked by inhibition of VEGFR-2, which is expressed in DRG and Schwann cells *(Sondell et al., 1999)*. VEGF can also stimulate migration of Schwann cells *(Schratzberger et al., 2000)*. These survival and migratory effects of VEGF suggest that it may counteract Schwann cell loss due to nerve injury and may enable newly formed Schwann cells to migrate to, and ensheath, nerve axons, thus promoting nerve regeneration. VEGF is vital for development, because the absence of a single VEGF allele results in an embryonic lethal phenotype in mice *(Ferrara et al., 1996)*. These early investigations of VEGF in the peripheral nervous system suggest its promise for therapy in neurodegenerative disease.

Growth Factors in Diabetic Neuropathy

In order to understand the pathogenesis of diabetic neuropathy, one must first consider the neurons that are at risk in this disorder and then address the qualities about these cells that make them vulnerable. In diabetic peripheral neuropathies, the symptoms always first appear in a stocking distribution. The vulnerability of these neurons is due to the length of their axons. Neurons that form the sensory relays of the spinal cord to the feet have the longest axons amongst any other neurons in the body. For illustration, the neuronal axon from the spinal cord to the foot in a tall individual may be over 1 m long. These long axon-bearing sensory neurons are the most vulnerable targets in diabetic peripheral neuropathy.

Having delineated the most degeneration-susceptible neuron pool in peripheral neuropathy, research has turned to consider how growth factors fit into this disease paradigm. Much attention has been given to 'the neurotrophic hypothesis', which states that developing neurons compete with each other for a limited supply of growth factors provided by target tissues. Neurons that successfully bind these limited growth factors will live, whereas the rest will die *(Yuen et al., 1996)*. In development, this selection serves to whittle down the initial overpopulation of neurons in the nervous system, such that only neurons making functional synapses will survive. However, the neurotrophic hypothesis may also have a bearing on degenerating cells in the disease state. In particular, the availability of growth factors in peripheral neuropathy may determine which vulnerable neurons will be able to survive the insult. Those neurons that do not have access to growth factors may not withstand the injury underlying diabetic peripheral neuropathy and thus degenerate. With this in mind, investigation of growth factors in peripheral neuropathy has focused on how growth factor supply and growth factor-mediated effects are altered in vulnerable peripheral neurons. In particular, research has examined how growth factor synthesis, transport, and cell-responsive signaling functions differ in the subset of vulnerable peripheral neurons during the diabetic disease state.

Expression and synthesis of growth factors

Alterations in the synthesis and expression levels of growth factors in the peripheral nervous system may account for the vulnerability of neurons and Schwann cells to the diabetic state. Of the growth factors discussed, most research has focused on the

changes in NGF synthesis and expression in *in vitro* and *in vivo* models of diabetic peripheral neuropathy. These models include injury-induced nerve damage such as sciatic nerve transection, axotomy or crush, the streptozotocin (STZ)-induced diabetic rat, and the spontaneously diabetic BB rat. Following sciatic nerve crush, NGF expression is upregulated threefold in L4 and L5 DRG. TrkA is also expressed by DRG during this time, suggesting that DRG mount a self-directed regeneration attempt *(Sebert and Shooter, 1993)*. After axotomy of adult rat sciatic nerve, NGF, TrkA, and p75 NGF receptors are 50% downregulated in DRG at 4–14 days post-injury and do not return to control levels until 30 days post-injury *(Krekoski et al., 1996)*. However, NGF, TrkA, and p75 are increased in Schwann cells distal to the sciatic nerve injury site, reaching maximal levels 5–7 days following the injury. In sciatic nerve injury models, NGF expression in Schwann cells is inversely related to axonal contact. For example, NGF expression increases in Schwann cells when proximal axons are degenerating, and expression is suppressed once regenerating axons grow in contact with the Schwann cells *(Taniuchi et al., 1986; 1988)*. TrkA expression follows a similar expression profile as NGF in Schwann cells of this model *(Bosch et al., 1989)*. These results suggest that the NGF system may be upregulated in Schwann cells in an attempt to promote their own survival and to provide NGF to nearby damaged neurons to promote their survival. Biopsies of human sural nerve recapitulate these results. NGF receptors are present in Schwann cells in sural nerves that contain degenerating axons, but these receptors are not detected in Schwann cells of nerves that have remyelinated *(Sobue et al., 1988)*.

Expression of the NGF system is also altered in rat models of diabetes. In STZ-diabetic rats, NGF levels are decreased in sympathetically innervated target organs, the superior cervical ganglion, and sciatic nerve *(Hellweg and Hartung, 1990)*. However, NGF expression levels returned to normal following allogeneic pancreatic islet transplantation that provides a physiological glucose homeostasis without immunosuppression. The diabetic condition itself causes NGF reduction and return to euglycemia can restore normal nerve function *(Hellweg et al., 1991)*. Decreased NGF production also occurs in genetically diabetic mice (C57Bl/KsJ db/db) *(Kasayama and Oka, 1989)*. There are little data about NGF expression in human diabetic patients, although these patients do exhibit increased NGF and TrkA mRNA in their lateral calf skin *(Terenghi et al., 1997; Diemel et al., 1999)*. This abnormal increase in skin may be the byproduct of a compensatory NGF production mechanism mounted to protect vulnerable NGF-responsive neurons. Furthermore, the p75 NGF receptor is upregulated in the sciatic nerves of patients with type 1 or type 2 diabetes mellitus *(Scarpini et al., 1996)*. However, others report depletion of skin NGF in patients with diabetic neuropathy, which correlates with decreased skin axon reflex vasodilation responses mediated by small sensory fibers *(Choi-Lunberg and Bohn, 1995)*. These authors suggest that skin-derived NGF, normally produced by keratinocytes, is important for maintaining nociception and thus loss of these cells and that NGF may lead to or exacerbate peripheral neuropathy. This controversy regarding the changes in expression of the NGF system in diabetes in humans is intriguing, because different responses may underlie the variable susceptibility to and symptoms of neuropathy between patients.

There is less information detailing the expression of classical neurotrophins other than NGF in diabetes. TrkB, the high-affinity receptor for BDNF and NT-4/5, and BDNF are increased in adult rat DRG following sciatic nerve damage, a model of peripheral nerve degeneration in diabetes *(Foster et al., 1994; Ha et al., 2001)*. Rats fed with galactose, which induces axonal atrophy and nerve conduction deficits similar to those seen in STZ rat models, exhibit increased BDNF protein in their peripheral nerves and muscles *(Mizisin et al., 1997a)*. BDNF levels are also increased in nerves of diabetic patients displaying axonal pathology, but interestingly, TrkB is decreased *(Sobue et al., 1998)*. The upregulated BDNF expression may be an attempt to restore tropic signaling by saturating the system with BDNF to compensate for diminished BDNF receptor levels.

The NT-3/Trk C system is altered in diabetic peripheral neuropathy. In rat models of diabetes, NT-3 mRNA is deficient in the leg muscles and treatment with insulin prevents the loss of NT-3 mRNA and protein. TrkC expression is also downregulated by 50% in diabetic rat DRG compared to normal age-matched controls *(Garofalo and Rosen, 1989; Fernyhough et al., 1998)*. However, there is controversy over NT-3 expression in diabetic rats. Although it is agreed that NT-3 protein expression is increased by 50% in the 12-week diabetic rats in the dorsal root and sural nerve, levels of NT-3 mRNA in the sural nerve are reported as increased in one study *(Cai et al., 1999)* but decreased in another *(Rodríguez-Pena et al., 1995)*. Therefore, care must be exercised in interpreting the role of endogenous NT-3 in peripheral neuron injury. Increased NT-3 expression, similar to BDNF, could represent a compensatory response to the loss of NT-3 in the target tissues that would normally be retrogradely transported back to the DRG cell body. Because NGF, BDNF, and NT-3 all mediate trophic responses in separate subsets of peripheral neurons, it is possible that the similar upregulation profiles represent a united effort to preserve the total neuron

population. The least explored neurotrophin in diabetic peripheral neuropathy is NT-4/5. There has been only one investigation of NT-4/5 to date, which found that this growth factor is decreased in the sciatic nerve of STZ-diabetic rats after 6 and 12 weeks *(Rodríguez-Pena et al., 1995)*.

There is a large literature describing the expression and synthesis of the IGF-I system in nerve injury and diabetic peripheral neuropathy models. Sciatic nerve crush in the rat increases IGF-I and IGF-II mRNA distal to the crush site, whereas IGF-I expression at the crush site is not increased until 4 days post-injury *(Pu et al., 1995)*. IGF-II expression is not altered at the crush site, but rather in more distal, intramuscular reaches of the nerves; this expression is lost after re-establishment of functional neuromuscular synapses *(Glazner and Ishii, 1995; Pu et al., 1995)*. Thus, IGF-II may be important in regenerating interrupted neuromuscular junctions *(Ishii, 1989; Glazner and Ishii, 1995)*. Furthermore, increased IGF-I, IGF-II, and IGF-IR expression are observed following sciatic nerve transection (Fig. 1) *(Cheng et al., 1996a; Hammarberg et al., 1998)*. During the first 3–7 days following sciatic nerve transection, the expressed IGF-I is localized mainly in Schwann cells of the intact nerve and the distal stump *(Cheng et al., 1996a; Zochodne and Cheng, 2000)*. IGF-I expression is also detectable in transected facial nerve 4–7 days post-transection, where it is mainly localized in astrocyte processes *(Gehrmann et al., 1994)*. Components of the IGF system are also altered in STZ-induced diabetes in the rat nervous system. In these rats, serum levels of IGF-I are reduced by 86% compared to control rats. IGF-I and IGF II mRNA transcripts are also decreased in the sciatic nerves of STZ-induced diabetic rats. Insulin treatment restored serum IGF-I peptide levels toward normality and inhibited transcriptional downregulation effects *(Olchovsky et al., 1991; Wuarin et al., 1994)*.

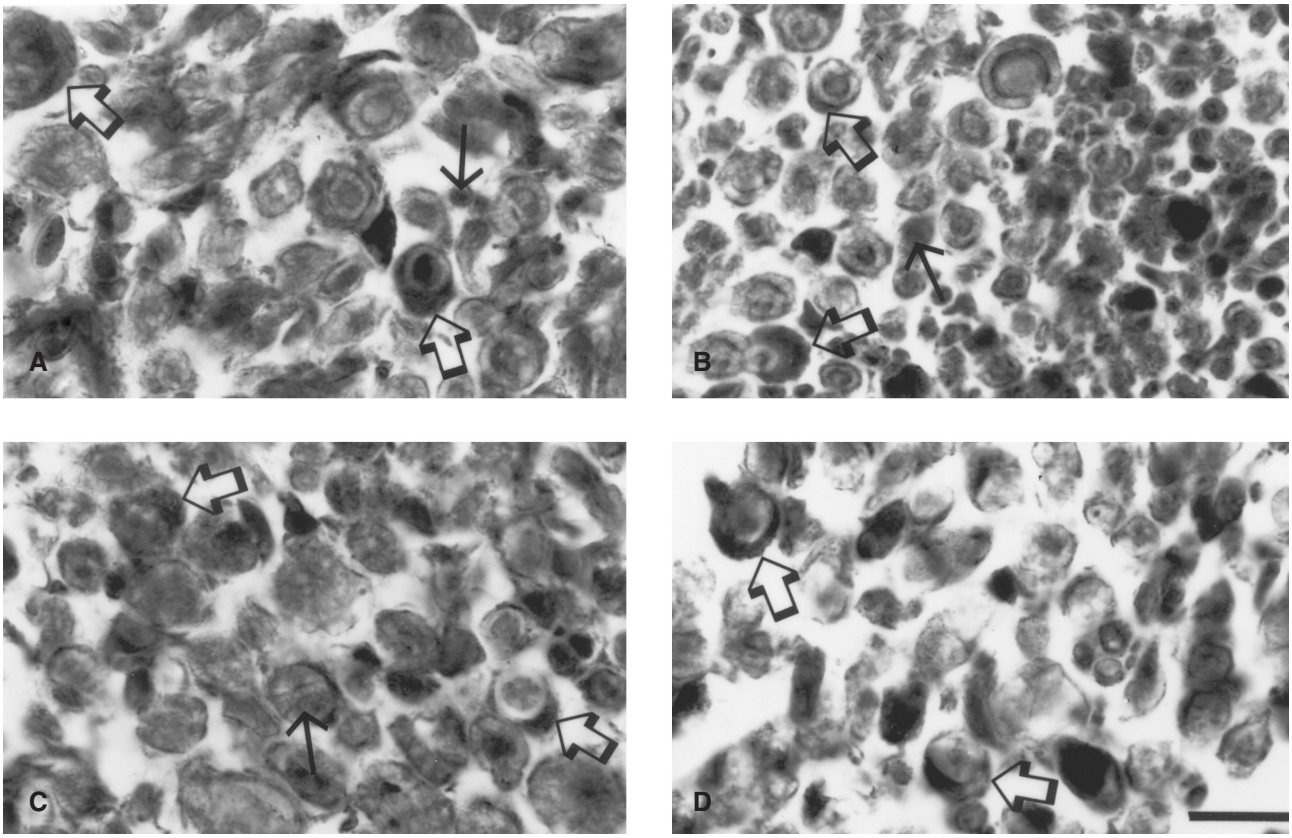

Figure1. Insulin-like growth factor-1 (IGF-I) expression after nerve transection. Rat sciatic nerves were transected, and IGF-I immunohistochemistry was performed 3 and 7 days after surgery using polyclonal antiserum for IGF-I. (A) IGF-I immunoreactivity in intact nerves. Schwann cells (black arrows) are stained but myelin sheaths (white arrows) are not. (B) IGF-I immunoreactivity at the distal stump at day 3. Axonal degeneration is observed (white arrow). Schwann cells (black arrows) and endoneurial sheaths (white arrows) are IGF-I positive. (C) IGF-I immunoreactivity in distal nerve stump at day 7. There is prominent axonal degeneration. IGF-I-immunopositive Schwann cells are wrapped around axonal debris (black arrow) or are proliferating (white arrows). (D) Longitudinal section of 7-day distal stump. Invading macrophages (white arrows) appear to be a major IGF-I source. Less IGF-I immunoreactivity remains in Schwann cells [reproduced with permission from Cheng *et al.*, *(1996b)*].

These experimentally diabetic rats also have reduced IGF-I and IGF-IR mRNA in the superior cervical ganglia and spinal cord *(Bitar et al., 1997; Bitar and Pilcher, 1998; Craner et al., 2002)*. In contrast, spontaneously diabetic obese Zucker (fa/fa) rats exhibit increased IGF-II mRNA in spinal cord and sciatic nerve, but IGF-I mRNA was not increased in the sciatic nerves of these rats *(Zhuang et al., 1997)*. Interesting differences in response to injury occur in different diabetes models. In the BB/W rat model of type 1 diabetes, IGF-I expression is upregulated in Schwann cells within 24 h following sciatic nerve crush injury. Expression of IGF-IR in this paradigm does not correspond with IGF-I. Yet, in the BB/Z rat model of type 2 diabetes, sciatic nerve crush promotes upregulation of IGF-I within 2 h in Schwann cells and this expression is synchronized with IGF-IR expression *(Ozdinler and Erzurumlu, 2001)*. Finally, in humans, IGF-I and IGF-IR levels are decreased in the serum of patients with diabetic peripheral neuropathy *(Migdalis et al., 1995)*.

Recent evidence suggests that expression of the cytokine-like growth factors CNTF and GDNF are also altered in models of peripheral neuropathy as well as in the human disease state. In a rodent nerve transection model, CNTF mRNA is downregulated and remains low, although in a nerve crush model, CNTF mRNA immediately decreases and recovers 4 weeks post-injury. However, the CNTFRα component of the CNTF receptor is increased following nerve injury *(Ito et al., 1998)*. Similar to the transection model, galactose-fed rats exhibit decreased CNTF-like bioactivity along with decreased CNTF protein, although mRNA levels were not affected *(Mizisin et al., 1997b)*. Furthermore, STZ-induced diabetic rats have a 70% reduction in CNTF-like bioactivity at month 2 *(Calcutt et al., 1992)*. This rodent pattern of CNTF expression is echoed in humans with varied peripheral neuropathies. CNTF levels are decreased in neurons, but the CNTFRα and LIFRβ components of the CNTF receptor are upregulated *(Ito et al., 2001)*. The CNTF levels inversely correlate with the extent of macrophage invasion of the diseased nerve, supporting the hypothesis that CNTF is an injury-induced growth factor *(Ito et al., 2001)*. Upregulation of the CNTF receptor components in response to peripheral nerve injury contrasts with the NGF, BDNF, and NT-3 systems that upregulate the growth factor rather than its receptor.

The expression pattern of GDNF has also been characterized in peripheral neuropathies of rats and humans. In the rodent sciatic nerve degeneration model, GDNF expression is upregulated in Schwann cells 48 h post-injury, peaking at week 1 and only returning to pre-injury levels after 6 weeks. GDNFRα expression also increases in Schwann cells following the peak in the level of GDNF and remains elevated for 6 months *(Höke et al., 2002)*. Patients exhibiting peripheral neuropathies caused by diseases other than diabetes also express increased GDNF and GDNFRα in the nerves. Expression of GDNFRα in these nerves correlates with both the degree of nerve axonal pathology and the extent of T cell and macrophage invasion *(Yamamoto et al., 1998)*, suggesting that changes in GDNF expression are a general hallmark of axonopathic disease, whereas CNTF expression changes are only noted in specific disease states.

Recent demonstrations that VEGF potently promotes regeneration have prompted investigation of the synthesis and expression levels of this growth factor in diabetic neuropathy. VEGF immunostaining of sciatic nerve and DRG from STZ-induced diabetic rats shows high VEGF levels in cell bodies and nerve fibers, although control animals have very little VEGF in these areas. Diabetic rats treated with insulin have intermediate VEGF levels between the uncontrolled hyperglycemic and non-diabetic rats, suggesting that control of the diabetic condition itself directly modulates the production of VEGF *(Samii et al., 1999)*. Little is known regarding VEGF expression in human diabetic peripheral neuropathy, although there is increased expression of VEGF in diabetic retinopathy *(Sueishi et al., 1996)*. Because VEGF is implicated in the pathogenesis of multiple complications in diabetes, increased VEGF expression may be a universal response to degrading cells and not specific to neuronal pathology.

Axonal transport and localization of growth factors

Neurotrophins exert survival and regenerative effects on both the neuron terminal, where they elicit neurite outgrowth or remodeling, and the cell body where they regulate transcription to alter overall cell function and structure. These dual signals are propagated to the cell bodies of peripheral neurons through retrograde signaling. Target-derived neurotrophins bind to receptors at neuron terminals and are then transported back through the axon to the neuronal cell body. Thus, initial binding at the axon induces local signaling at the axonal terminal, while retrograde transport allows for delayed neurotrophin signaling at the cell body *(Ginty and Segal, 2002)*. Recent evidence supports the 'signaling endosome model' in retrograde signaling. In this mechanism, target-derived neurotrophin binding to Trk receptors first induces receptor-activated signaling in the local neuron terminal. This local signaling then causes the neurotrophin–receptor complexes to be internalized by endocytosis. Some of these endosomes re-configure within the cell, so that activated Trk receptors are within the vesicle

membranes and the neurotrophin is within the lumen of the vesicle. Such an orientation maintains the activated Trk tyrosine kinase domains in the cytoplasm, where they can interact with downstream-signaling effectors during transport through the axon, and ultimately, at the cell body. In the case of NGF binding to Trk A, the endocytotic retrograde transport system will permit both quick signaling at the nerve terminal, a sustained signaling through axonal transport, and a long-lasting signal that is still active by the time the endosome reaches the cell body. For excellent reviews of recent data supporting the endocytosis model in retrograde neurotrophin signaling, refer to Ginty and Segal *(2002)*.

Although the mechanism of neurotrophin retrograde transport is only recently reported, there is ample evidence that retrograde transport is altered in the diabetic state. NGF transport is depressed in the mesenteric nerves of STZ-induced diabetic rats; these nerves subserve the alimentary tract and may be prone to distal axonopathy induced by diabetic insult *(Schmidt et al., 1983)*. Interestingly, decreased retrograde transport in the mesenteric nerves is present before any axonal pathology, and the levels of depressed transport did not worsen even when axonal degeneration did occur *(Schmidt et al., 1985)*. STZ-induced diabetic rats also show reductions of 50% in NGF transport via the sciatic nerve, and NGF receptor saturation decreased by 45% compared to control rats. Thus, diabetic peripheral neuropathy also suppresses axonal transport in somatic sensory neurons *(Jakobsen et al., 1981; Hellweg et al., 1994)*.

Very little is known about axonal transport of the other neurotrophins during diabetes. STZ-diabetic rats exhibit decreased anterograde and retrograde transport of endogenous BDNF in the sciatic nerve. However, the significance is unclear, because these same rats maintain normal ability to transport radio-labeled BDNF injected into sciatic nerve *(Mizisin et al., 1999b)*. Similarly, NT-3 retrograde and anterograde transport through sciatic nerve is decreased in diabetic rats. Due to this suppression, neurotrophic support of the large caliber sensory neurons subserved by axons of the sciatic nerve is blunted in peripheral neuropathy *(Fernyhough et al., 1998)*. Diminished axonal transport of neurotrophins is not a reflection of decreased neurotrophin levels, because axonal transport of enzymes (acetylcholinesterase and choline acetyltransferase), cytoskeletal components (tubulin and actin), and other proteins are also decreased in the nerves of diabetic rats *(Schmidt et al., 1975; Medori et al., 1985; Fink et al., 1987)*. The general depression of axonal transport in diabetes inhibits normal neuronal function by blocking cell body access to signaling molecules and structural proteins from the axon terminal. Because the cell body is unable to receive growth factor-mediated signals, it does not mount a survival or regenerative response. As such, the rescue attempts mounted by surrounding cells are inhibited by depressed axonal transport, thus further compromising the already impaired neurons.

Growth factors and signal transduction in diabetes

Growth factors promote survival and regenerative effects on neurons by initiating signal transduction cascades within the cells, which can ultimately reach the nucleus. Here, genes may be upregulated or downregulated by the growth factor-promoted signal to generate a response to environmental stimuli or damage. Recent research has defined the intracellular signaling initiated by growth factor binding to healthy neurons, although there is little information available about growth factor-induced signaling in diabetes. However, the levels of intracellular growth factor signaling likely parallel the levels of growth factor expression. Thus, the altered growth factor expression profiles in diabetic peripheral neuropathy as discussed above yield insight into how normal growth factor signaling may be altered in this disease.

Recent studies of neurotrophin signaling, particularly NGF, have focused on the spatial logistics, delineating the separate signal cascades induced by growth factors first at the axon terminal and then at the cell body following retrograde transport (see Fig. 2 for an overview of neurotrophin signaling). Both signal cascades are initiated by NGF binding to TrkA receptors, which causes receptor dimerization and autophosphorylation of intracellular tyrosines *(Kaplan et al., 1991)*. Signaling is mediated by members of the Ras/extracellular signal-related kinase (ERK) pathway, the phosphatidylinositol 3 kinase (PI3K) pathway, and the phospholipase-C-γ1 (PLC-γ1) pathway *(Patapoutian and Reichardt, 2001)*. The cascade of events begins when Shc binds to the activated TrkA receptor and becomes phosphorylated *(Dikic et al., 1995)*. Phosphorylated Shc then recruits the Grb-2/son of sevenless (SOS) complex to the membrane. This bound and activated Grb-2/SOS complex then stimulates activation of the Ras protein *(Rozakis-Adcock et al., 1992)*. Ras can then activate the p38 MAPK/MAPK-activating protein kinase 2 pathway and Raf that, in turn, activate the ERK pathway. There are many members of the ERK family. Amongst those activated by NGF at the axon are ERK1, ERK2, and ERK5 *(Atwal et al., 2000; Watson et al., 2001a)*. The ERKs activate neuronal transcription factors, such as the ribosomal S6 kinases (RSKs) and the CRE-binding protein (CREB). Blocking either the p38 or ERK pathways decreases CREB activation, but blocking both simultaneously completely abolishes CREB activation. Thus, both of these NGF-initiated pathways modulate

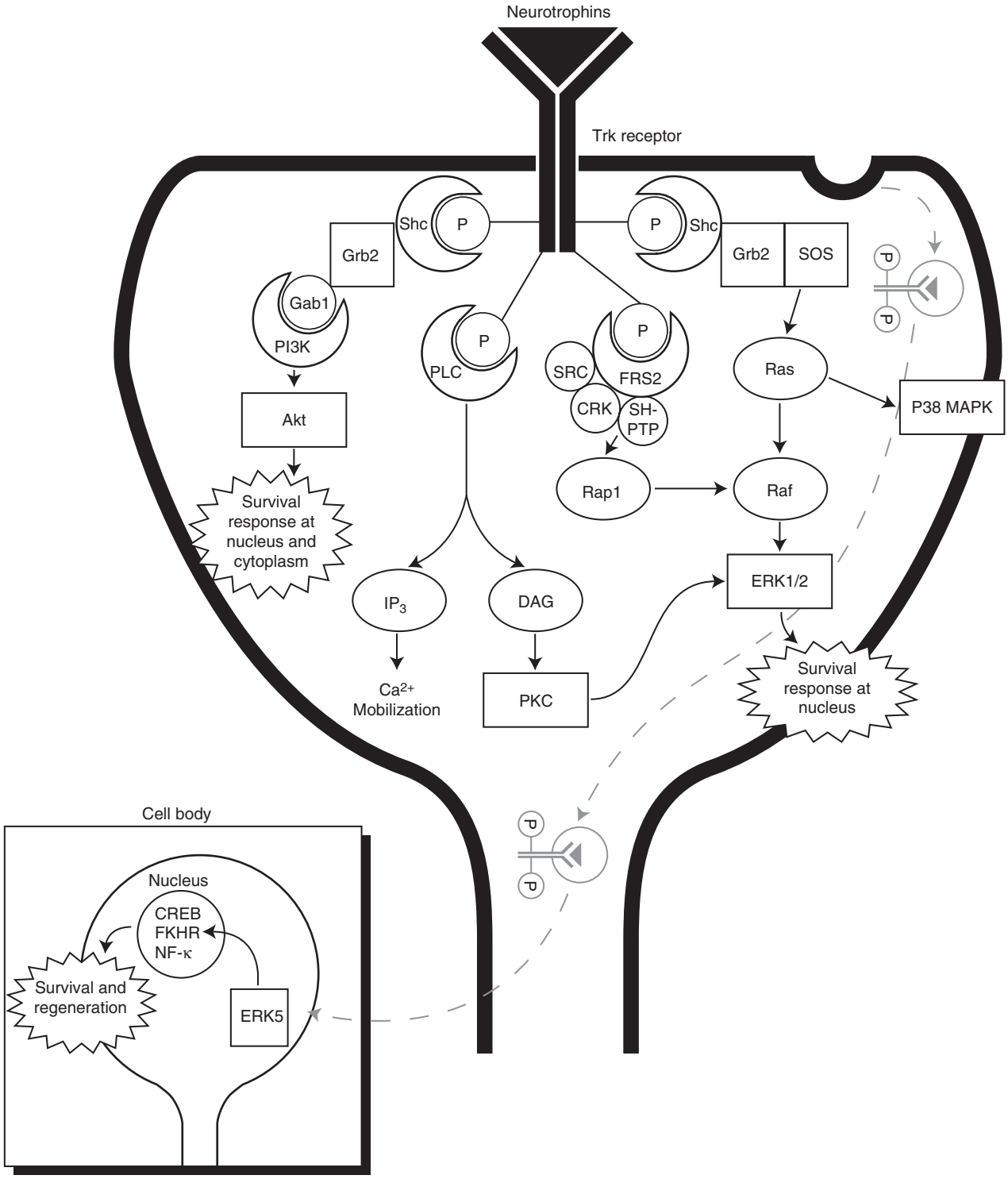

Figure 2. Neurotrophin-signaling pathways in neurons. Neurotrophin binding at Trk receptors causes phosphorylation of intracellular receptor tyrosines. Shc binds to these phosphorylated residues and can thereby activate the phosphatidylinositol 3 kinase (PI3K) and MAPK pathways. The PI3K and MAPK pathways both transduce survival signals to the neuron. Also, receptor endocytosis (follow gray figures to inset) allows for MAPK activation at the cell body (via ERK5) and further activation of nuclear transcription factors. Thus, retrograde transport of endocytosed Trk receptors promotes neuronal survival and regeneration. Neurotrophins also mediate activation of PLC-γ and FRS-2 when these proteins bind to phosphorylated tyrosine residues of Trk receptors. The significance of these pathways in neuronal survival and regeneration is less clear than that of the PI3K and MAPK pathways.

signaling at the transcriptional level *(Xing et al., 1996; 1998)*.

Although not demonstrated in primary neurons, the ERK pathway can also be initiated by fibroblast growth factor receptor substrate-2 (FRS-2) adaptor molecule binding to the activated TrkA receptor. FRS-2 then recruits Grb-2, Crk, the protein phosphatase Src homology protein tyrosine phosphatase-2 (SH-PTP), and Src. The FRS-2/Crk complex is known to induce activation of Rap-1, which activates Raf, and thereby activates ERK signaling. Pathways downstream of FRS-2 may be involved in PC12 differentiation, suggesting a potential role for FRS-2 pathways in axon repair and outgrowth in injured primary cells *(Meakin et al., 1999; MacDonald et al., 2000)*. Additionally, the FRS-2/Src complex may be important in retrograde signaling, because Src family tyrosine kinases are implicated in receptor endocytosis *(Wilde et al., 1999)*.

In addition to activating the p38 MAPK and ERK pathways, Ras also activates the PI3K pathway in response to NGF. Like ERK, PI3K signaling begins with binding of Shc to the phosphorylated Trk receptor, which then recruits Grb-2. Activated Grb-2 acts as a docking site for the protein Gab-1. Gab-1 then recruits PI3K, which initiates downstream activation of the serine–threonine kinase Akt. Akt activates several transcription factors operative in survival and regenerative responses, such as the forkhead-transcription factors (FKHRs), CREB, and NF-κB *(Holgado-Madruga et al., 1997; Zheng et al., 2002)*. Akt also regulates cytoplasmic proteins that are important for cell survival. For example, Akt phosphorylates the pro-apoptotic protein Bad, causing it to bind to the 14-3-3 proteins and thus prohibiting free Bad from exerting apoptotic effects on the mitochondrial membrane *(Datta et al., 1997; 1999)*. The Rho family of GTPases are also activated by PI3K signaling, and these mediate axonal branching *(Ozdinler and Erzurumlu, 2001)*. Thus, PI3K may modulate survival and axonal morphology.

A final pathway activated by Trk receptors is the PLC-γ1 pathway. Phosphorylated Trk receptors bind and activate PLC-γ1. The PLC-γ1 enzyme then hydrolyzes phosphatidylinositides, generating inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG). The former product can induce intracellular Ca^{2+} mobilization, whereas the latter activates protein kinase C (PKC). PKC activation may also activate ERKs and, thus, can induce survival and regenerative responses *(Rhee, 2001)*. In summary, NGF-TrkA signaling can influence receptor complex internalization via ERK, axonal morphology via PI3K, and survival and regenerative responses at the cytoplasmic and nuclear levels via ERK, PI3K, and PLC-γ1. CNTF and GDNF also utilize the same ERK, PI3K, and PLC pathways *(Takahashi, 2001)*.

Even then, how are these signaling pathways at the axon unique from those at the cell body? As discussed previously, the NGF-TrkA complex is internalized into endosomes and is retrogradely transported through the axon to the cell body. TrkA receptors are oriented in these endosomes with their cytoplasmic domains outside the vesicle, such that they can activate other signaling molecules during transport. Signaling substrates such as PLC-γ1, Rap-1, Ras, ERK1/2, and PI3K can all bind to vesicle-bound TrkA, as described by Howe *et al. (2001)*. Thus, TrkA can initiate signaling of the ERK, PI3K, and PLC-γ1 pathways within the axon while being transported back to the cell body. The ERKs are modulated differently at the axon and at the cell body in response to NGF. Although local neurotrophin stimulation of sensory neuron distal axons activates ERK1 and ERK2, these are not activated in the cell body. Instead, ERK5 is activated at the cell body. ERK5 activity induces CREB that enhances neuronal survival *(Watson et al., 2001b)*. Thus, it appears that NGF-TrkA compartmentalization into endosomes permits selection between signals that are relayed quickly back through the axon (ERK5) and those that function at the axon and have limited effect at the cell body (ERK1/2). The significance of ERK spatial signaling is not yet fully understood. Neurons may utilize spatial signaling to combat neurodegenerative effects. Rapid signals at the axon that are mediated by receptor binding may be attempts to regulate or regenerate compromized axonal morphology. Long-term signals carried to the cell body then allow the neuron to mount a second wave of survival mechanisms through the whole cell, including inhibiting apoptotic effects and producing additional cytoskeletal components. This spatial difference in signaling is exclusively observed by the ERK family and not by PI3K or PLC-γ. Ongoing research will be vital for understanding these processes in both normal and diseased systems.

IGF-I signaling in neurons is well characterized, and it utilizes the same ERK and PI3K pathways activated by the neurotrophins (Fig. 3). The pathways begin with IGF-I-mediated phosphorylation of IGF-IR intracellular receptor tyrosines *(De Meyts et al., 1994; O'Connor et al., 1997)*. As in the NGF system, Shc binds to the activated receptor and is phosphorylated there *(Giorgetti et al., 1994; Kim et al., 1998a)*. Shc can then bind the adaptor protein complex, Grb2/SOS, leading to activation of the Ras/MAPK pathway *(Ravichandran, 2001)*. This ultimately leads to increased ERK phosphorylation. Additionally, and unlike NGF, IGF-I can initiate intracellular signaling through insulin receptor substrate-1 and -2 (IRS-1 and -2). The IRS proteins bind to phosphotyrosine residues of the IGF-IR and act as docking proteins, allowing binding of

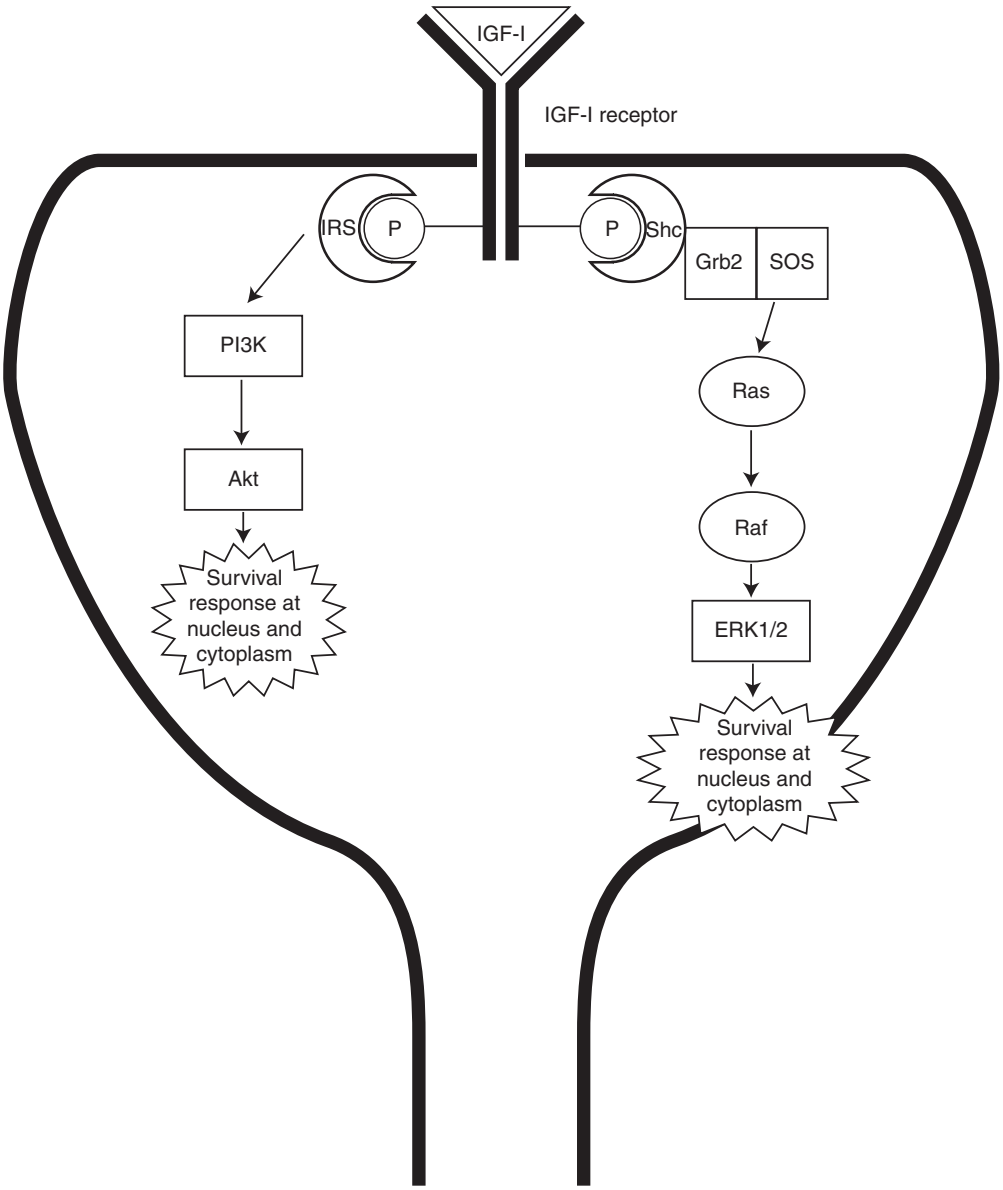

Figure 3. Insulin-like growth factor-1 (IGF-I)-signaling pathways in neurons. IGF-I binding to the IGF-IR causes phosphorylation of intracellular receptor tyrosines. When Shc binds to these tyrosines, it activates a cascade of proteins that ultimately activates the MAPK pathway. IRS binding to a phosphorylated IGF-IR tyrosine leads to activation of the phosphatidylinositol 3 kinase (PI3K)/Akt pathway. Both the MAPK and PI3K pathways initiate survival responses in neurons.

additional downstream-signaling molecules that contain Src homology 2 domains *(Myers and White, 1996)*. This enables binding of PI3K *(Giorgetti et al., 1993; Carpenter and Cantley, 1996; Kim et al., 1998b)*, which activates Akt *(Dudek et al., 1997; Vanhaesebroeck et al., 1997)*. Akt signaling is exerted on the membrane by potentiating L subtype calcium channels *(Blair et al., 1999)*, upregulating the anti-apoptotic proteins Bcl-2 and Bcl-xL *(Pugazhenthi et al., 2000; Chrysis et al., 2001)*, and preventing the anti-apoptotic proteins Bax and Bad from localizing to the mitochondria *(Bai et al., 1999; Gleichmann et al., 2000; Yamaguchi and Wang, 2001; Vincent et al., 2002)*. Furthermore, Akt induces nuclear changes by activating transcription factors, including the FKHRs *(del Peso et al., 1999; Zheng et al., 2000; Suhara et al., 2002)* and NF-κB *(Heck et al., 1999)*.

The ERK, PI3K, and PLC-γ1 pathways are induced by growth factors in normal neurons, but how are these pathways affected in neurons in a diabetic environment? Diabetes decreases the levels of NGF, TrkA, TrkB, NT-3, CNTF, and IGF-I in neurons, suggesting that the signaling pathways mediated by these growth factor systems are inhibited and thus neurons

will be more susceptible to further damage. There are reports of increased ERK phosphorylation in the DRG, but not in the sural nerve, of diabetic rats *(Fernyhough et al., 1999)*. In the DRG of diabetic rats, ERK and p38 MAPK are activated at week 8. Furthermore, total p38 and c-Jun NH_2-terminal kinase (JNK) proteins are increased in the sural nerves from type 1 and type 2 diabetic patients *(Purves et al., 2001)*. p38 and JNKs are activated in response to stress, and hence, upregulation in diabetic nerve is a sign of neuronal distress. Perhaps the upregulation of ERK is an attempt to override this stress signal. Similarly, activation of ERK and JNK are increased in astrocytes following partial sciatic nerve ligation, indicating that an amplified ERK response to injurious JNK activation may be a general survival mechanism *(Ma and Quirion, 2002)*. Yet, increased ERK activation correlates with the onset of hyperalgesia in STZ-diabetic rats and inhibition of ERK blocks static allodynia *(Ciruela et al., 2003)*. Further studies are required to clarify the role of ERK activation in the nervous system during diabetes.

In STZ-diabetic rats, fast transport of activated p38 and JNK occurs in sciatic nerve at a similar rate as retrograde and anterograde transport. NT-3 not only prevents the activation of JNK and p38 but also their transport *(Middlemas et al., 2003)*. At present, there are no data detailing alterations in Akt or PLC-γ1 signaling in the peripheral nervous systems of diabetic *in vitro* or *in vivo* models. Active PI3K and Akt are reduced in the vagus nerve of STZ-diabetic rats, suggesting that this signaling cascade is compromized in the nervous system in diabetes *(Cai and Helke, 2003)*. However, in order to appropriately develop targeted therapies for alleviation of peripheral neuropathy, it is key to understand how the normal signaling of neurons and glia are changed by diabetes. Clearly, more work is necessary to clarify the roles of growth factor-induced signaling within this disease.

Growth Factors as Therapeutics in Peripheral Neuropathy

Growth factor treatments have been proposed in many neurological degenerative diseases, including Huntington's Disease, Alzheimer's Disease, Parkinson's Disease, and ALS *(Yuen and Mobley, 1995; Gozes, 2001; Hurelbrink and Barker, 2001; Alberch et al., 2002)*. The potential of growth factors as therapeutics to prevent or alleviate diabetic complications has also been addressed in recent years *(Apfel, 1999; Chiarelli et al., 2000)*. Initial encouraging results of investigations of growth factors in *in vitro* and *in vivo* models of diabetic neurons, however, have been tempered by clinical trials of NGF in the treatment of human diabetic peripheral neuropathy. Yet, greater understanding of the pathogenesis of peripheral neuropathy continues to suggest that growth factor treatments could be beneficial, and that the dose, mode of delivery, and a lack of combination therapies all may have limited the efficacy of previous trials. The following sections will address the newly interpreted pathogenesis of diabetic neuropathy and will survey the *in vitro*, *in vivo*, and clinical trial results of growth factor therapy in this disorder.

The pathogenetic mechanisms underlying diabetic neuropathy

The cellular mechanisms underlying nervous system complications in diabetes have proved elusive. Many pathways are implicated in the development of vascular, retinal, renal, and nerve complications, although none has emerged as a clear pathogenetic link. Instead, certain cellular pathways feature more prominently in specific diabetic complications than in others, and inhibition of any one pathway does not prevent all of these disorders. This in itself sums up the difficulty in studying diabetic complications, they are disparate and multifactorial. Four pathways associated with the hyperglycemic environment are strongly implicated in the pathogenesis of diabetic complications. These are the polyol pathway, the advanced glycation end-product (AGE) pathway, the protein kinase C (PKC) pathway and the hexosamine pathway. Interestingly, recent evidence suggests that each of these four pathways result in the same common pathway of cellular injury mediated by oxidative stress. In order to understand how the four pathways converge on one mechanism of injury, it is important to first define each mechanism.

The polyol pathway is a minor mechanism for glucose utilization in normal cells but becomes prominent in cells exposed to hyperglycemia. The key enzyme of the polyol pathway, aldose reductase, has low affinity for glucose, and hence, is only activated in a hyperglycemia. When activated, aldose reductase converts glucose to sorbitol with the consumption of one molecule of NADPH *(Petrash et al., 1994)*. The sorbitol is, in turn, oxidized to fructose by sorbitol dehydrogenase using NAD^+ as a cofactor *(Jeffery and Jornvall, 1983)*. Activity of the polyol pathway then increases the $NADH : NAD^+$ ratio but decreases the cytoplasmic NADPH pool. Reduced availability of NAD^+ suppresses the activity of other glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) that also require NAD^+ as a cofactor. Inhibition of GAPDH decreases the normal flux of glucose through the glycolysis pathway and also leads to the accumulation of GAPDH metabolites that then activate the hexosamine pathway *(Sirover, 1999)*. NADPH is critical to the regeneration of reduced glutathione (GSH) that is the most important cellular

antioxidant *(Mates et al., 2002)*. Thus, although shunting glucose through the polyol pathway is an initial attempt to reduce intracellular glucose levels, this pathway ultimately results in loss of both normal energy production and protective systems. Aldose reductase inhibitors prevent diabetic neuropathy in both animal models and in clinical trial *(Engerman et al., 1994; Obrosova et al., 2002)*.

AGEs are increased in diabetic tissues, and hence, may promote complications including neuropathy *(Thornalley, 2002)*. AGEs result from auto-oxidation of glucose followed by a cascade of decomposition to reactive dicarbonyls that, in turn, react with amino groups on both intracellular and extracellular proteins *(Wautier and Guillausseau, 2001)*. AGE-modified proteins have impaired properties due to alterations of their tertiary structure. AGE-modified proteins can also bind to AGE receptors (such as RAGE), which causes formation of reactive oxygen species and activation of the apoptotic transcription factor, NF-κB *(Brownlee, 2000)*. Inhibiting the formation of AGEs inhibits the development of diabetic nephropathy *(Brownlee, 2000; Forbes et al., 2001; Agardh et al., 2002)*, but there is no evidence suggesting that inhibition of AGE formation is therapeutic for diabetic peripheral neuropathy.

The PKC pathway is activated by DAG. Because DAG is synthesized in response to hyperglycemic conditions, PKC pathway activation is increased in response to high glucose. This increase in PKC pathway flux has been linked to vascular, retinal, renal, and cardiovascular diabetic complications *(Koya and King, 1998)*, and some *in vitro* work suggests that PKC may figure in peripheral neuropathy as well *(Cotter et al., 2002; Eichberg, 2002)*. Specifically, PKC activation induces abnormalities in blood flow and also promotes activation of the NF-κB transcription factor *(Ishii et al., 1996; Bursell et al., 1997)*. Complications in blood flow could interrupt access of nerves to ample blood supply, and thus, could secondarily cause neuronal damage. However, there is no direct evidence that inhibition of PKC has a therapeutic effect in human peripheral neuropathy or neuronal degeneration.

The hexosamine pathway is activated when glycolytic intermediates accumulate in hyperglycemic milieu. One intermediate, fructose-6-phosphate, is converted to glucosamine-6-phosphate and then to UDP-*N*-acetylglucosamine through successive enzymatic reactions. The *N*-acetylglucosamine components of the UDP-*N*-acetylglucosamine are enzymatically joined to serine and threonine residues of proteins, thereby producing proteoglycans and O-linked glycoproteins. Many of the acylglycosylated proteins are transcription factors that increase proteins associated with diabetic complications such as TGF-β1 that promotes nephropathy and plasminogen-activator inhibitor-1 (PAI-1) that inhibits normal blood clotting and increases vascular complications *(Sharma and McGowan, 2000; Carr, 2001)*. Interestingly, PKC is required for PAI-1 upregulation, suggesting synergy between the pathways leading to diabetic complications *(Goldberg et al., 2002)*. The hexosamine pathway is particularly implicated in type 2 diabetes. Over-expression of the rate-limiting enzyme of the hexosamine pathway, fructose-6-phosphate amidotransferase, causes cells to become insulin resistant and promotes hyperinsulinemia, mimicking changes in type 2 diabetes *(McClain and Crook, 1996)*.

Each of the four pathways described produces oxidative stress (Fig. 4), as does the over-activation of the mitochondrial electron transport chain in hyperglycemic cells. These processes, how they lead to oxidative stress, and their relationship to diabetic complications are described in an excellent review *(Brownlee, 2001)*. Accumulation of reactive oxygen species (ROS) through all of these mechanisms is severely detrimental to the cell. Unchecked ROS produce (1) lipid, DNA, and protein peroxidation *(Greene et al., 2000)*, (2) ischemia and reduced nerve blood flow *(Cameron and Cotter, 1997)*, and (3) cellular apoptosis *(Russell et al., 1999)*. These oxidative insults are particularly deleterious in nerves, which then suggests a fundamental mechanism for diabetic neuropathy.

Emerging evidence supports the role of ROS in the pathogenesis of peripheral neuropathy. Sensory neurons treated with high glucose respond with a peak in ROS production, followed by loss of regulation of mitochondrial membrane potential and activation of caspases (Fig. 5) *(Russell et al., 2002)*. The STZ-induced diabetic rat also exhibits oxidative stress in peripheral nerves, leading to caspase-3 activation and apoptosis of DRG neurons *(Schmeichel et al., 2003)*. Antioxidant therapy of diabetic rats increases the mitochondrial oxidative state and decreases the impairment of digital nerve conduction velocities, suggesting that suppression of diabetes-induced oxidative stress can inhibit the progression of peripheral neuropathy *(Stevens et al., 2000; Ueno et al., 2002)*. Thus, this early data supports the underlying mechanism of oxidative stress in diabetic neuropathic disease. Therefore, any examination of therapeutics in diabetic peripheral neuropathy should analyze the proposed therapeutic in the context of this oxidative stress mechanism.

Although it is generally agreed that oxidative stress contributes to the pathogenesis of diabetic peripheral neuropathy, there is debate on how oxidative stress morphologically impacts upon neurons. In particular, although some groups suggest that hyperglycemia and oxidative stress cause neuronal death by apoptosis *(Russell et al., 2002; Schmeichel et al., 2003)*, others

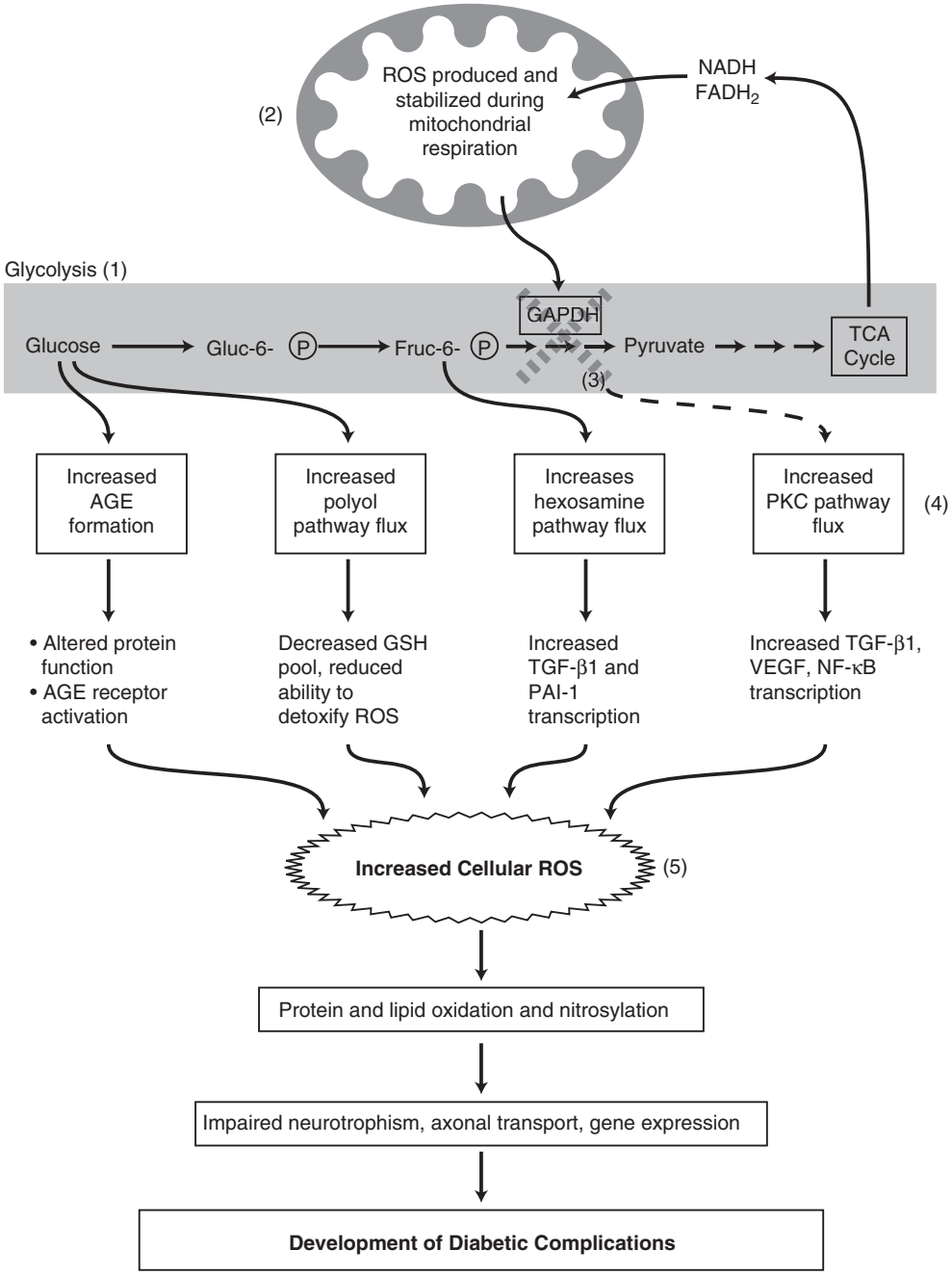

Figure 4. The pathogenesis of diabetic complications. The hyperglycemic state promotes oxidative stress and diabetic complications via five steps. Step 1: increased glucose due to the hyperglycemic state is funneled into glycolysis and the tricarboxylic acid (TCA) cycle, yielding NADH and $FADH_2$. Step 2: the NADH and $FADH_2$ are shuttled into the mitochondria, and through electron transport, ATP is produced. A byproduct of this process is increased reactive oxygen species (ROS) production and stabilization. Step 3: ROS inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), thereby inhibiting glycolysis past this point. As a result, glucose and glycolytic precursors, such as glucose-6-phosphate, fructose-6-phosphate, and diacylglycerol (DAG), build up. Step 4: increased glucose and glycolytic precursors lead to increased flux through the advanced glycation end-product, polyol, hexosamine, and protein kinase C (PKC) pathways. These pathways lead to various alterations in transcription and protein function, which result in increased cellular ROS (Step 5). Increased ROS promote protein and lipid damage, impair neurotrophism, axonal transport, and gene expression, and ultimately promote the development of diabetic complications.

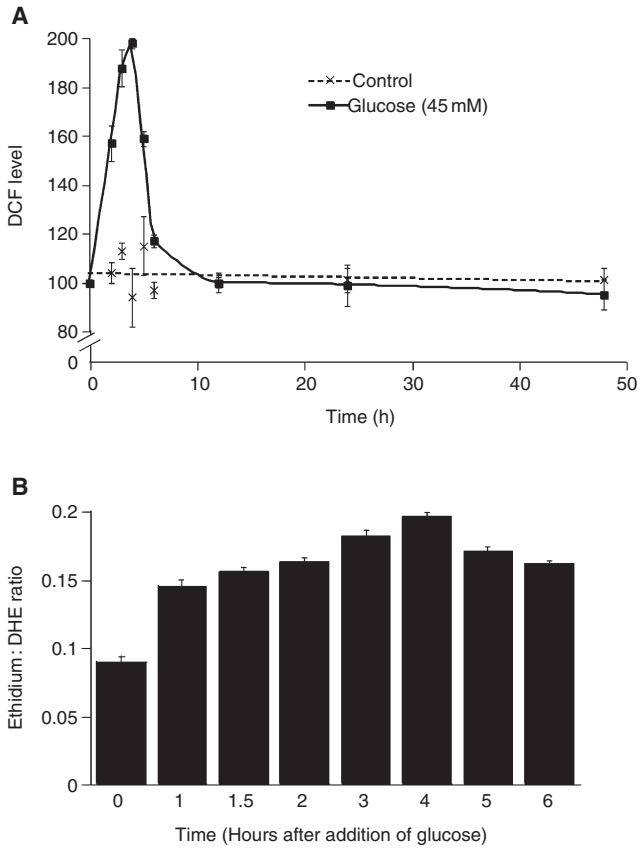

Figure 5. High glucose induces reactive oxygen species (ROS) production. (A) Cultured embryonic rat dorsal root ganglia (DRG) were treated with ±20 mM added glucose for up to 48 h in media containing 2 μM of CM-H_2DCFDA. In the presence of ROS, CM-H_2DCFDA is oxidized to fluorescent 2′7′-dichlorofluorescein (DCF) in the neuron. DCF was measured using confocal microscopy. Results were obtained from five experiments standardized against control values at 0 h. With added glucose, results are expressed as percentage control at each time point (1–48 h). In control media (containing 25 mM glucose), results during 1–48 h are expressed as percentage control 0 h. There is no change in control values over 48 h. In 45 mM total glucose, there is an initial increase in the production of ROS (mean DCF levels) that peaks during 3–4 h, then declines, corresponding to increased neuronal death. (B) Cultured embryonic rat DRGs were exposed to 45 mM glucose for 0–6 h + 3 μM dihydroethidium (DHE). DHE is converted to ethidium by oxidation and is another means of measuring ROS production. DHE oxidation to ethidium was measured using fluorimetry to determine superoxide production during peak glucose-induced ROS generation. Results are expressed as the ethidium : DHE ratio [reproduced with permission from Russell *et al.*, *(2002)*].

contend that there is no evidence of neuronal apoptosis in experimental diabetic models *(Schmidt, 2001; Cheng and Zochodne, 2003)*. The contradictory evidence in rat models of diabetes concerning apoptotic death, or lack of it, may be due to the duration of diabetes and the time points at which nerve samples were analyzed. Schmeichel *et al. (2003)* report activation of caspase-3 and apoptosis in the DRG after 1 and 3 months of diabetes, but not after 12 months. The authors speculate that, in the onset of diabetes, neurons are susceptible to apoptotic death but that the remains of these neurons are cleared from the ganglia by 12 months. Additionally, the remains of degenerating axons are found in long-term diabetic models. Peripheral neuron axons are very long and span a significant distance in the body, and hence, require a more spatially concerted effort to be cleared compared to cell bodies in the DRG. This may be why degenerated neuronal processes are evidenced long after cell bodies are removed. This could account for the appearance of neuroaxonal dystrophy (NAD) evidenced in diabetic models, with no evidence of apoptotic cell bodies *(Schmidt, 2001; Cheng and Zochodne, 2003)*.

However, if hyperglycemia induces oxidative stress and hence apoptosis in peripheral neurons, we might expect to consistently observe apoptotic death throughout the long-term diabetes rather than the first 1–3 months only. Perhaps, hyperglycemic induced oxidative stress preferentially targets a subset of neurons which are lost early in the disease, and the remaining neurons are more resistant, leaving little or no detectable apoptosis at later time points. Indeed, the largest neurons of DRG are particularly vulnerable in diabetic rats compared to the more numerous small neurons *(Kishi et al., 2002)*.

Moving away from the concept that it is hyperglycemia that produces diabetic neuropathy, there is an alternative hypothesis suggesting that the loss of insulin is a more critical mediator of the disorder. In the Goto–Kakizaki rat model of diabetes, peripheral nerve abnormalities increase in parallel with the decrease in insulin *(Murakawa et al., 2002)*. Additionally, rat models of type 1 diabetes have decreased IGF-I, TrkA, and β-tubulin proteins after nerve crush compared to that seen in type 2, hyperinsulinemic rats *(Pierson et al., 2003a)*. Treatment of the type 1 diabetic rats with proinsulin C peptide, which enhances insulin signaling without decreasing the blood glucose levels, reverses the inability to regenerate nerve fibers following a crush injury *(Pierson et al., 2003b)*. Similarly, the loss of insulin may produce the decreased mitochondrial membrane potential in STZ-diabetic rats *(Huang et al., 2003)*. Suboptimal insulin levels inhibit diabetes-induced changes in neurons, likely due to the neurotrophin-like effects of insulin. Insulin and IGF-I activate similar downstream signal transduction pathways, and IGF-I has significant neuroprotective actions in the peripheral nervous system, discussed earlier. The reduced insulin hypothesis does not necessarily conflict with the hyperglycemia hypothesis because it is likely to be the balance between neurotrophic

support and the degree of injury that ultimately determines the ability of a neuron to survive in a stressed environment. Therefore, the combined roles of decreased insulin, hyperglycemia, and oxidative stress will require careful analysis before we can fully understand how these contribute to the onset and progression of diabetic neuropathy.

Distinct from direct effects on neurons, the diabetic state also may produce neuropathy through cardiovascular disease *(Resnick and Howard, 2002)*. Diabetic patients commonly experience hypertension and atherosclerosis, two conditions that are also exacerbated by oxidative stress *(Di Carli et al., 2003)*. The end result of diabetic cardiovascular disease includes ischemia of the nerves that will promote oxidative nerve damage and contribute to neuropathy. Although this is an important area for consideration in the development of diabetic neuropathy, this literature is reviewed elsewhere and would take us beyond the scope of this discussion of growth factors that directly promote nerve cell survival or regeneration.

In vitro evidence of growth factor treatment in peripheral neuropathy

The mechanisms of growth factor signaling in hyperglycemic neurons are just beginning to be explored. The advantage of *in vitro* systems is the ability to explore temporal effects of high glucose on gene expression and protein regulation in the presence or absence of growth factors, changes that are not easily detected in *in vivo* models or human-diseased nerves. Importantly, *in vitro* models must demonstrate that high glucose does not induce deleterious effects due to osmolar effects rather than through the metabolic pathway. Increased D-glucose in the cell media of differentiated PC12 cells (twofold to sixfold above normal media) results in cell death, but the isomer L-glucose does not affect PC12 cell viability. Thus, high glucose treatment does not inflict effects via osmolar stress, and hence, is a good model for studying cellular abnormalities in response to hyperglycemia *(Koshimura et al., 2002)*. Treating with 20–25 mM glucose in addition to the glucose concentration in normal media is analogous to the rises in blood glucose that can be experienced by a diabetic patient following a meal. Cultured rat neurons from the superior cervical ganglion exposed to high glucose have reduced neurite growth, reduced neurite caliber, and retraction of neurite growth cones compared to normo-glycemic treated neurons. However, addition of IGF-I prevents these effects *(Russell and Feldman, 1999)*. Cultured rat DRG neurons treated with high glucose also exhibit neurite degeneration and activation of caspase-3, leading to apoptotic death. These effects are also counteracted by IGF-I (Fig. 6) *(Russell et al., 1999)*. IGF-I also prevents glucose-induced apoptosis of cultured rat Schwann cells, via activation of the PI3K pathway *(Delaney et al., 2001)*. Thus, IGF-I is capable of preventing hyperglycemic damage and promotes survival of sympathetic and sensory neurons as well as their supporting Schwann cells. However, the IGF-I-signaling mechanisms that promote survival in these hyperglycemia models are yet to be fully understood. Additionally, the roles of other growth factors have yet to be assessed in hyperglycemic *in vitro* models. Such investigations could prove important in mining the potential effectiveness of growth factors for prevention of diabetic neuropathy.

Growth factors and animal models of diabetes

Rodent models of diabetes have proved crucial for investigating the pathogenesis and effects of diabetic neuropathy. STZ-treated rats are the most commonly employed *in vivo* model for studies of diabetes and its complications *(Szkudelski, 2001)*. In this model, administration of NGF decreases the development of structural and behavioral deficits in the peripheral nervous system. The therapeutic benefits include preventing increased pain thresholds, preventing decreased sensory nerve amplitudes in feet, maintenance of myelin thickness, and upregulation of substance P and calcitonin gene-related products in diabetic DRG *(Apfel et al., 1994; Fernyhough et al., 1995; Unger et al., 1998; Goss et al., 2002)*. Furthermore, treatment of STZ rats with 4-methylcatechol, a stimulator of NGF production, results in increased NGF content, nerve conduction velocities, and larger myelinated nerve fibers and axon diameters compared to untreated diabetic rats *(Hanaoka et al., 1994)*. DRG cultured from STZ mice also respond to NGF treatment with higher substance P and calcitonin gene expression than diabetic controls *(Sango et al., 1994)*. In the genetically diabetic db/db mouse, treatment with NGF returns the expression of substance P and calcitonin to the levels observed in wild-type mice *(Schmidt et al., 1995)*. NGF treatment not only prevents neuronal injury in diabetes *(Elias et al., 1998)* but also aids the regeneration of neuronal fibers *(Whitworth et al., 1995)*. NGF also can decrease neuropathic pain, exemplified in a model of sciatic nerve transection *(Jubran and Widenfalk, 2003)*. Because patients with diabetic neuropathy often experience limb pain, the ability of NGF to preserve nerve function and decrease associated pain could provide significant clinical benefit.

In addition to NGF, the other neurotrophin family members have therapeutic effects in the peripheral nervous system of diabetic rodents. Exogenous BDNF treatment of galactose-fed rats promotes structural improvements and inhibits the decreased motor neuron conduction velocity typical of peripheral

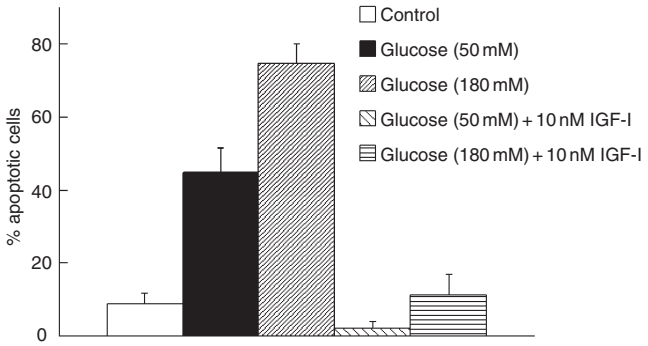

Figure 6. Insulin-like growth factor-1 (IGF-I) prevents glucose-induced apoptosis in dorsal root ganglia (DRG) neurons. Rat embryonic DRG neurons were cultured for 24 h in defined serum-free medium (containing 30 mM glucose), followed by treatment with 50 mM total glucose, 180 mM total glucose, 50 mM total glucose with 10 nM IGF-I, and 180 mM glucose with 10 nM IGF-I, all for a further 24 h. The concentration of glucose indicates the total concentration of glucose present in the medium. The percentage of apoptotic neurons increases in a dose-dependent fashion with increasing concentrations of glucose. IGF-I reduces the percentage of apoptotic neurons in the presence of high glucose, even up to 180 mM glucose ($p < 0.001$) [reproduced with permission from Russell *et al.*, *(1999)*].

neuropathy. However, BDNF is not capable of attenuating sensory neuron structural changes or the neuropathic deficit in sensory nerve conduction velocity *(Mizisin et al., 1997a)*. In mice subjected to sciatic nerve ligation, BDNF/Trk B may be responsible for neuropathic pain-like thermal hyperalgesia following mechanical peripheral nerve injury *(Yajima et al., 2002)*. Thus, BDNF may not be beneficial for repairing sensory deficits secondary to nerve injury and could exacerbate neuropathic symptoms. BDNF ameliorates other diabetic-induced metabolic changes including disrupted glucose and lipid metabolism in obese diabetic animals. Also, BDNF improves the response to exogenous insulin in STZ-diabetic mice and, in some models, even reduces blood glucose concentration *(Nakagawa et al., 2000; Ono et al., 2000; Tsuchida et al., 2002)*. If these effects of BDNF bear out in continued study, researchers will have to consider the potential effects of circulating BDNF treatment on the nervous system. In galactose-fed rats, BDNF alone inhibits the deficit in motor neuron conduction velocity, although NT-3 administration ameliorates the sensory nerve conduction velocity deficit with little effect on the motor deficit *(Mizisin et al., 1998)*. However, both the motor and sensory nerve conduction velocity deficits in STZ-diabetic rats are normalized by treatment with NT-3 *(Mizisin et al., 1999a)*. NT-3 provides the additional benefit of preventing the accumulation of neurofilaments in large diameter sensory neurons of diabetic rats and thus may improve neuronal transport and function *(Sayers et al., 2003)*. Thus, it may be possible to utilize the neuron-selective actions of BDNF and NT-3, along with the particular functional deficit they can correct, as a joint therapy in peripheral nervous disease.

The IGFs, GDNF, and VEGF also have been tested in animal models of diabetic neuropathy. Both IGF-I and IGF-II prevent hyperalgesia and improve sensory nerve regeneration in STZ rats *(Zhuang et al., 1996)*. IGF-I also reverses NAD in the superior mesenteric ganglion and in the mesenteric nerves of these rats, implicating IGF-I in functional preservation during complications of the autonomic nervous system *(Schmidt et al., 1999)*. GDNF administration improves distal sciatic nerve afferents into the dorsal horn of STZ mice *(Akkina et al., 2001)*. Additionally, diabetic mice treated with GDNF exhibit increased cutaneous innervation and axon branching in the flank and footpad skin compared to non-treated diabetic mice. These data contrast with NGF treatment that increases axonal branching but does not improve cutaneous innervation *(Christianson et al., 2003)*. Few studies have incorporated VEGF, possibly due to concerns that this growth factor can promote tumor growth and diabetic eye disease *(Antonetti et al., 1999; Funatsu et al., 2002)*. Constitutive expression of VEGF-1 and VEGF-2 by gene transfer improves both the vascularity and the function of the large and small fibers in *ex vivo* peripheral nerves *(Schratzberger et al., 2001)*. The non-physiological experimental design of this study, however, does not provide strong evidence that VEGF would produce similar benefit in an animal or clinical treatment paradigm. Other treatments with growth factors via adenoviral infection are also yielding promising results in STZ rats. For example, intramuscular injection of NT-3-expressing adenovirus prevents slowing of motor and sensory nerve conduction velocities *(Pradat et al., 2001)*.

Although the growth factors described show some ability to alleviate or prevent peripheral neuropathic changes, these results must be interpreted with caution and should not be extrapolated directly to the human disorder. The rodent nervous system is not a perfect model for human pathogenetic progression, but it does hint the promise of growth factors. Still, mechanistic elaboration of growth factor signaling in the diabetic rodent models would be helpful for understanding the efficacy of growth factor treatments, and thus, much work remains to be performed in these models.

Growth factors as therapeutics for peripheral neuropathy in human clinical trials

The potent effects of growth factors on neurons fueled early speculation that they might be useful in treating human nervous system diseases. Reduced expression and transport of growth factors in animal models of

diabetes and in human patients suggest that these deficits mediate diabetic neuropathy. Then, by extension, reintroduction of the growth factors may prevent the development of the disease. Cell culture and animal models of the disease have provided strong evidence to support the initiation of clinical trials. NGF, as the first discovered growth factor and the most tested in experimental diabetes models, was the first to be studied in the clinical setting. A three-phase clinical trial was completed in 2000. Intradermal injections of 1–3 μg of recombinant human NGF (rhNGF) are considered safe and tolerable to patients. Symptoms associated with the treatments are minor, including localized tenderness of NGF-injection sites and slight discomfort in the wrist and deep structures (Dyck *et al., 1997)*.

In phase 1–2 of the rhNGF trial, 250 patients were recruited at 15 US study sites. The study included a placebo group receiving equal volumes of vehicle buffer. Two experimental groups received either 0.1 μg/kg rhNGF or 0.3 μg/kg rhNGF. All subjects received subcutaneous injections (rotated amongst the hands, feet, and abdomen) three times per week over 6 consecutive months. The most reported side effect was injection site hyperalgesia, which was mild to moderate. Subjects also reported general myalgias. At the study conclusion, global symptom assessment showed a strong beneficial effect of rhNGF treatment on the patients' perception of their neuropathic systems, although partial patient unblinding during the trial complicates interpretation of this result. However, both cooling detection threshold and the quantitative neurologic examinations (NISSLs) were improved in rhNGF-treated patients compared to the placebo-control group. Overall, the study was determined to show preliminary evidence for efficacy in disease treatment, although the authors stipulated that the small size of the study group and brief duration of monitoring may have underestimated the beneficial effects *(Apfel et al., 1998)*.

The subsequent phase 3 trial was a 12-month double-blind and placebo-controlled study with 505 patients from 84 outpatient centers. In this trial, the treatment group received 0.1 μg/kg of rhNGF by subcutaneous injection 3 times per week. The lower dose from the phase 1–2 trial was selected to minimize injection site discomfort. As before, these patients had type 1 or 2 diabetes mellitus with stable glycemic control and were diagnosed with diabetic peripheral neuropathy in the absence of any other confounding systemic disease. Patients receiving rhNGF had more secondary adverse effects than the placebo group, including myalgia, peripheral edema, and most often reported, injection site pain/hyperalgesia. However, contrary to expectations following the phase 1–2 trial, the phase 3 trial did not demonstrate therapeutic benefit of rhNGF treatment in diabetic peripheral neuropathy *(Apfel et al., 2000)*. Alternative clinical trials of rhNGF in HIV-associated sensory neuropathy demonstrated significant improvements in pain symptoms when patients received similar does of rhNGF twice a week for 18–48 weeks *(McArthur et al., 2000)*. There may be differential factors between the diabetic peripheral neuropathy phase 3 trial and the phase 1–2 and HIV-associated neuropathy clinical trials, which could account for the efficacy of the latter and not the former. For example, one of the main endpoint determinants of peripheral neuropathy improvement, the NISSL, was not sensitive to small fiber sensory dysfunction, which is the main neuronal population that probably responds to NGF. Also, the rhNGF used in the phase 3 trial was manufactured differently and was supplied in an altered buffer solution compared with the phase 1–2 study. The 0.1 μg/kg of dose is probably at the threshold of its effective concentration, and combined with the altered production methods, this could have decreased the bioavailable concentration below its range of efficacy. Thus, there are many potential avenues to explain the disappointing outcome of the phase 3 trial and its inconsistency with the phase 1/2 data and the HIV trials.

The potential of other growth factors as therapeutics in diabetic peripheral neuropathy is also being investigated. A recent double-blind placebo-controlled clinical trial of recombinant human BDNF also did not show any measurable improvement in diabetic neuropathy, although the dosage was both safe and tolerable for patients *(Wellmer et al., 2001)*. NT-3 was examined in healthy subjects and concluded to have tolerable side effects, but the phase 1 trial using NT-3 to treat diabetic and chemotherapy-induced neuropathy was discontinued in 1997. No report of this study was ever published, suggesting that the results were not favorable *(Chaudhry et al., 2000)*. Currently, a phase 1–2 trial of VEGF in diabetic peripheral neuropathy is underway. In contrast to the subcutaneous injections utilized in other growth factor clinical trials, VEGF is being delivered by gene transfer. Naked VEGF DNA will be injected intramuscularly into affected extremities (foot, calf muscle, or distal thigh) in a dose escalation paradigm over 4 years. Results of this study should be available in 2006 *(Isner et al., 2001)*.

To date, there are no clinical data using IGF-I, CNTF, or GDNF in diabetic neuropathy. Although CNTF has been tested in ALS clinical trials without any identified success, it could have some efficacy in motor symptoms of diabetics *(ALS CNTF Treatment Study Group, 1996)*. One clinical trial of IGF-I in ALS has shown a trend toward functional improvement, suggesting that it could have similar beneficial effects on motor neurons in diabetic neuropathy *(Lai et al.,*

1997). Because IGF-I is also neurotrophic for sensory peripheral neurons, it may be an excellent candidate for therapeutic study in diabetic peripheral neuropathy, although more preliminary work in animal models is warranted before undertaking a clinical examination. Importantly, it will be critical to consider drug delivery models for any future growth factor clinical trials in neurodegenerative disease. Previous delivery mechanisms, such as systemic administration and internal pumps, have not been beneficial; flooding the body with a growth factor is not advantageous. Because of the specificity for growth factors and particular sets of neurons, targeted growth factor delivery to key degenerative sites is a better therapeutic paradigm. Thus, the early positive results of viral vector delivery of growth factors and the outcome of the current VEGF study may demonstrate alternative methods for more efficacious dose delivery, and thus, potential enhancement of growth factor effects *(Thoenen and Sendtner, 2002)*.

Summary and Future Directions

Growth factor research throughout the last 50 years has produced ample evidence of the survival and regenerative effects of these agents in the neuronal system. Early studies documenting altered levels and altered retrograde signaling in diabetic animal models and in humans have prompted investigation of these growth factors in treatment of diabetic peripheral neuropathy. Although early therapeutic trials were not uniformly positive, new insight into the pathogenetic mechanism underlying diabetic peripheral neuropathy is leading to a revised investigation of growth factors in this disease. In this pursuit, studies detailing the effects of growth factors on oxidative stress mechanisms in diabetic animal models will be key. Also, better understanding of growth factor-directed signaling as well as the spatial logistics of signaling will be crucial for selecting the most efficacious growth factor or growth factors. In parallel, progress must also be made in drug delivery mechanisms, so that the appropriate growth factor can be tailor-delivered to the appropriate subset of neurons and glia at risk. Thus, there are many research frontiers yet to be explored in growth factor studies, and the anticipated end result is a superior therapy for diabetic peripheral neuropathy.

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