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Regulation of Neurotrophic Signaling Molecules in Motor Neurons, Primary Sensory Neurons and Target Tissues in Senescence

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"Success is not final, failure is not fatal;
It is the courage to continue that counts."

—Winston Churchill

To My Parents and My Family

ABSTRACT

A hallmark of senescence is sensorimotor impairment, characterized by gait disturbance, deficits in postural control, and muscle weakness. The object of this thesis work was to elucidate some of the molecular basis underlying these disturbances, mainly referring to the occurrence of characteristic changes in gene-expression pattern, especially, alterations in trophic signaling associated with the phenotypic changes seen in motoneurons as well as primary sensory neurons and target muscles during aging.

Based on our previous findings of trk downregulation in aging DRG and spinal motoneurons that may reflect a failure of targets to supply neurotrophic factors, the expression of neurotrophins in target tissues and peripheral nerves of aged rats was examined by use of RT-PCR. In target muscles, decreased levels of NGF, BDNF, NT3, NT4 mRNAs were observed, and notably, the decrease co-varied with the extent of behavioural sensorimotor disturbances among the aged individuals. In contrast, the peripheral nerve of aged rats showed a reciprocal regulation of NGF, BDNF and NT4 mRNAs. These aging-related changes provide evidence of an attenuated neurotrophin-trk-signaling in senescence, possibly relating to a breakdown in the neuron-target interaction, which may explain some of the phenotypic changes of senescent neurons.

In contrast, a dramatic upregulation of GDNF mRNA was detected in target muscles and to a lesser extent also in peripheral nerve during aging. A parallel increase of the preferred receptors, GFRα1 and c-Ret, in primary sensory neurons and motoneurons strongly indicates an enhanced GDNF signaling in senescence. The phenomenon of an increased GDNF signaling between the target muscles and motoneurons may serve as a compensatory mechanism in the context of decreased neurotrophin-trk signaling in aged motoneurons, promoting axon regeneration, sprouting, and muscle fibre re-innervation. Since GDNF has been suggested to act primarily on unmyelinated PSN, an increased GDNF-GFRα1/Ret signaling and a decreased neurotrophin siganling mutually may clarify many of the senescent phenotypic changes of primary sensory neurons, and may explain why unmyelinated primary afferents are better preserved during aging.

A slight upregulation of FGF-2/FGFR1 signaling was established, whereas the gp130 signaling components (CNTF, IL-6, LIF, CNTFRα, LIFR, IL-6R or gp130) were maintained at young adult level in the aged spinal cord, indicating that FGF/FGFR1 signaling may be involved in the phenotypic changes of aged motoneurons but the gp130 cytokines continue to support motoneurons at about the same level throughout life. In the target muscles, there were a marked downregulation of IL-6 and FGF-2 mRNAs and an upregulation of FGFR1, CNTFRα as well as gp130. It is suggested that these regulations reflect the repair situation in senescent muscles.

The changes in cytokine expressions showed an increased IFN-γ signaling and in parallel an increase of other cytokines as IL-1β, IL-6 and TGF-β1 in senescent spinal cord, which may provide evidence of the involvement of inflammation during aging, marked by gliosis associated with axon dystrophy, myelin aberrations and synaptic disconnection.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Ming Y.**, Bergman E., Edström E. and Ulfhake B. Reciprocal changes in the expression of neurotrophins in target tissues and peripheral nerves of aged rats *Neuroscience Letters 1999; 273(3): 187-190*

II. Bergman E., Kullberg S., **Ming Y.** and Ulfhake B. Upregulation of GFRα-1 and c-RET in primary sensory neurons and spinal motoneurons of aged rats J. Neurosci. Res. 1999; 57(2): 153-165

III. **Ming Y.**, Bergman E., Edström E. and Ulfhake B. Evidence for increased GDNF signaling in aged sensory and motor neurons Neuroreport 1999; 10(7): 1529-1535

IV. Edström E. *, **Ming Y.** *, Kullberg S. and Ulfhake B. Alterations during aging in the expression of FGF2 signaling components and the family of gp130 signaling molecules in the spinal cord, skeletal motoneurons and target muscles
* both authors contributed equally to this study
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V. Kullberg S. *, **Ming Y.** *, Edström E. and Ulfhake B. Changes in expression of cytokines in the spinal cord of aged rats: evidence for increased INF-γ signaling
* both authors contributed equally to this study
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ABBREVIATIONS

ART	artemin
BDNF	brain-derived neurotrophic factor
FGF-2	basic fibroblast growth factor
CGRP	calcitonin gene-related peptide
CNTF	ciliary neurotophic factor
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFRα	GDNF family receptor α
GPI	glycosyl-phosphatidyl inositol
IL-6	interleukin-6
IL-1β	interleukin-1β
IFN-γ	interferon-γ
IR	immunoreactivity
-LI	-like immunoreactivity
LIF	leukemia inhibitory factor
MHC I	major histocompatibility complex class I
NGF	nerve growth factor
NT3	neurotrophin-3
NT4	neurotrophin-4
NTN	neurturin
$p75^{NTR}$	p75 neurotrophin receptor
PSP	persephin
RT-PCR	reverse transcriptase-polymerase chain reaction
SP	substance P
trk	tropomyosin related kinase
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α

1. INTRODUCTION

"Aging seems to be the only available way to live a long life."
—Daniel Francois Esprit Auber

1.1. Senescence

Senescence, the state of being old, can be defined as an inexorable process characterized by the increased probability of death with age and the occurrence of characteristic phenotypic changes in multicellular organisms due to a decline of biological functions and of the organism's ability to adapt. (Johnson et al., 1999). Historically, plausible theories on the mechanisms of aging have been divided into two general categories: one pinpointing intrinsic biological clocks or "programs" (genetic), and the other emphasizing extrinsic or environmental interactions that damage cells and organs (epigenetic) (Warner et al., 1987; Martin and Baker, 1993). These are not mutually exclusive. The programmed theories hold that a timetable will regulate gene expressions of cells as well as the functions of organs, e.g. endocrine system and immune system, and consequently control the pace of aging. In contrast, the latter includes cell deterioration, malfunctions and ultimately cell death due to reactive oxygen species (ROS) generated by metabolism, accumulation of somatic mutations, damaged proteins and lipid peroxide. Importantly, aging has many facets and thereby no single theory can be a host of all the phenomena of aging. It is likely to assume that the pace and natural history of aging be affected by the combination of genetic and epigenetic factors.

1.1.1. Oxidants, oxidative stress and Aging

In the multifactorial process of aging, there is considerable evidence indicating that the generation of ROS, metabolites of molecular oxygen, and the response to oxidative stress are key factors in determining longevity. Reactive oxygen species, including superoxide anions, hydroxyl radicals and hydrogen peroxide, have been found to damage various cell components and to trigger the activation of specific signaling pathways, which can influence numerous cellular processes linked to aging and aging-related diseases (Finkel and Holbrook, 2000). The burden of ROS production is largely counteracted and regulated by an intricate antioxidant defense system, e.g. catalase, superoxide dismutase (SOD) and glutathione peroxidase. The balance between ROS production and antioxidant defenses determines the degree of oxidative stress that may consequently induce modification to cellular protein, lipids and DNA. The amount of oxidative damage to these molecules has been shown to

increase exponentially during aging in a variety of tissues in different species (Sohal and Weindruch, 1996). In addition, the efficacy of antioxidant defenses that maintain physiological homeostasis may decrease with advancing age (Sohal and Brunk, 1992; Sohal and Orr, 1992).

1.1.2. Caloric restriction and Aging

As yet, there seems no way to be able to retard the human aging process. Caloric restriction (CR), referring to a dietary regimen low in calories without undernutrition, is the only exception. CR has been shown to extend life span in a wide range of organisms including rodents, and also to slow the progression of a variety of aging-related diseases (Masoro, 2000; Suzuki et al., 1997). The CR regimen also prevents age-associated declines in psychomotor and spatial memory tasks (Ingram et al., 1987) and loss of dendritic spines necessary for learning (Moroi-Fetters et al., 1989) and improves the brain's plasticity and ability for self-repair (Mattson, 2000). One possible biological mechanism underlying the anti-aging action of CR has been hypothesized that CR could attenuate age-associated oxidative damage by either enhancing antioxidant defences or by promoting repair of damaged molecules (Masoro, 2000). Another mechanism that has been suggested is that CR may slow down accumulation of potentially harmful/aberrant abnormal proteins by speeding up protein turnover (Aksenova et al., 1998; Dhahbi et al., 1999).

1.1.3. Aging-related sensory and neuromuscular dysfunctions

With advancing age, sensorimotor functions as well as cognitive processes become impaired, which seriously compromises the daily activities of many elderly people (Lamberts et al., 1997; Larish et al., 1988; Woollacott, 1986). Certain neurological symptoms, e.g. deficits in postural control, alterations in gait cycle, muscle weakness and unsteadiness, are so common in healthy elderly human populations that they are regarded as part of 'normal aging'. A similar range of behavioural defects are encountered in aging rodents (see Appendix), suggesting that rodents may serve as a useful model in trying to elucidate the mechanisms underlying these disturbances. Skin and skeletal muscle, targets of sensory and motor neurons, show characteristic features of aging, e.g. dermal atrophy, muscle fiber loss and atrophy in muscle (Caccia et al., 1979). Many of these changes have been suggested to be of neurogenic origin (Tomonaga, 1977). Innervation deficits during aging have been considered a consequence of an almost 'facultative' loss of neurons. However, in contrast to

neurodegenerative diseases, neuron loss is not as extensive as once thought (Monji et al., 1994; Coleman and Flood, 1987; Morrison and Hof, 1997) and does not show a strong co-variation with functional impairment during aging. Instead, aging is manifested by loss of neuronal connections, axon dystrophy, myelin aberrations, phenotypic changes in gene expression pattern, and neuron atrophy in certain cell populations.

1.1.4. General background of the study

To investigate the mechanisms underlying the aging-related aberrations, we use rats with impairment of sensory and motor functions as revealed by standardized behavioral tests (Appendix). For primary sensory neurons (PSN), there is a small unselective loss of neurons, a selective cell body atrophy of large myelinated sensory neurons, abundant axon aberrations, and phenotypic alterations in the expression pattern of neuropeptdes and neurotrophic receptors (Bergman et al., 1996; Bergman et al., 2000; Bergman and Ulfhake, 2000; Bergman et al., 1999a; Bergman et al., 1999b; Bergman and Ulfhake, 1998; Fundin et al., 1997). Sensory neurons of aged rats disclosed a dramatic decrease in calcitonin gene-related peptide (CGRP) and substance P (SP) affecting mainly small (B4 binding) sensory neurons (Bergman et al., 1996; Bergman et al., 1999a). In large myelinated (RT97 positive) sensory neurons of the same individuals, a novel expression of galanin and a robust upregulation of neuropeptide tyrosine (NPY) are evident (Bergman et al., 1996; Bergman et al., 1999a). This pattern of changes may have relevance for the observation that aging individuals are affected by disturbed proprioception/exteroception than by changes in nociception. The characteristic size distribution of neurotrophin receptor (trk) expressing sensory neurons is maintained in the aged rats, with a selective expression of trkA and trkC in small and large sensory neurons, respectively and trkB expressed by neurons of all size categories (Bergman et al., 1996; Bergman et al., 1999b). However, the level of trk expression in aged sensory neurons is downregulated and, furthermore, a covariation between the distribution of symptoms and trk-downregulation is evident with the sensory neurons innervating the hind limbs being more affected than those of the face (Bergman et al., 1999b). Changes in neurotrophin expression in target skin during aging show a pattern consistent with the regulation of trk receptors in PSN (Bergman and Ulfhake, 2000).

For motoneurons, there is also a distinct pattern of phenotypic changes in the aged rats, with small loss of motoneurons duing aging (~15% at 30 month of age). Age motoneurons have a preserved cholinergic phenotype and cell body size (Johnson et al., 1993; Johnson et

al., 1999a), but disclose a highly specific pattern of regulation of neuropeptides and neurotrophic receptors. Both CGRP and gowth-associated protein 43 (GAP43) are maykedly upregulated in the aged motoneurons, a regulatory pattern typical of growth and regeneration (Johnson et al., 1995). Besides, aged motoneurons show a robust downregulation of trkC and trkB mRNA, suggesting target muscle neurotrophin deficiency. Based on the background, it is undoubtedly intriguing to provide further insight into regulation of neurotrophic signaling, particularly changes of neurotrophins or other neurotrophic factors during aging.

1.2. Neurotrophic Factors

Trophic factors, with the literal meaning of "nourishment", are defined as molecules that support survival and growth of neurons and their processes. In case of target-derived neurotrophic factors, once synaptic targets are established, neurons become dependent on their targets to provide specific signaling molecules, referred to as neurotrophic factors, for continued survival and differentiation. During the development of the CNS, approximately half of the neurons undergo apoptotic death (Raff et al., 1994). The survival and subsequent maintenance of neuronal integrity requires continued suppression of the intrinsic apoptotic machinery by, e.g. target-derived neurotrophic mechanisms (Henderson, 1996). As the central concept of the neurotrophic factor hypothesis, targets of nerve innervations were postulated to secret limiting amount of survival factors that exert effects to ensure a balance between the size of a target organ and the number of innervating neurons (Purves, 1988; Hamburger et al., 1981; Oppenheim, 1991).

The neurotrophic factors were found to regulate survival, differentiation, normal growth and maintenance of neurons in the peripheral and central nervous systems. Target-derived neurotrophins are captured in nerve terminals by receptor-mediated endocytosis and transported retrogradely to the cell bodies where they exert their neurotrophic effects (Korsching, 1993). Accumulated evidence for nonclassical trophic interactions has suggested that signaling by neurotrophic molecules seems not only to be restricted to the retrograde transport mode (DiStefano et al., 1992) but may include paracrine mode of action, autocrine signaling (Schecterson and Bothwell, 1992), and anterograde axonal transport (Altar and DiStefano, 1998; von Bartheld et al., 1996; Zhou and Rush, 1996).

The following families of molecules has been established to possess neurotrophic functions: the nerve growth factor (NGF) family, the glial cell line-derived neurotrophic factor (GDNF) family, the fibroblast growth factor (FGF) family, and the neuropoietic

cytokines, such as ciliary neurotrophic factor (CNTF) and leukaemia inhibitory factor (LIF). The discovery of these families of neurotrophic factors, the characterization of their receptors and at least partially, and the revelation of the downstream signaling pathways have enabled us to explain a wide range of phenomena in the development, maintenance, as well as plasticity and repair of the nervous system. Importantly, cell-cell communication represents the combined effects of many factors such that unlike in vitro studies where cell lines are treated with single factors, the growth and survival of cells in vivo are under the influence of simultaneous actions of many polypeptide factors including neurotrophic factors, which may interact with one another to form a complicated network of trophic activities with a high degree of pleiotropism and a considerable overlap in biological activities (Korsching, 1993).

1.3. NGF Family

1.3.1. Ligands and Receptors

The nerve growth factor (NGF) family of neurotrophins (NTs) is a group of structurally and functionally related proteins that exert profound effects on neural development, survival, function and plasticity, which consists of NGF (Scott et al., 1983; Ullrich et al., 1983; Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT3) (Maisonpierre et al., 1990) and neurotrophin-4/5 (NT4/5) (Hallböök et al., 1991; Berkemeier et al., 1991). The members of NGF family show binding specificity for particular receptors that belong to the tropomyosin-related kinase (trk) family of tyrosine kinase receptors (Bothwell, 1991): NGF binds to trkA (Klein et al., 1991; Kaplan et al., 1991), while both BDNF and NT4 act by binding to trkB (Squinto et al., 1991; Soppet et al., 1991), and NT3 interacts primarily with trkC (Lamballe et al., 1991) but also with trkA and trkB (Cordon-Cardo et al., 1991; Soppet et al., 1991). Non-catalytic isoforms, lacking the intracellular tyrosine kinase domain, have been identified for trkB and trkC. These truncated trk receptors may act as negative regulators of neurotrophin receptor activities (Eide et al., 1996) or to restrict the diffusion of cognate ligands from sites of release (Biffo et al., 1995). The findings suggest that the specificity of neurotrophin effects and the vast majority of biological responses are mediated through trk receptor signaling (Barbacid, 1994; Lewin, 1996; Snider, 1994; Bothwell, 1995). The neurotrophin receptor p75 ($p75^{NTR}$), a distant member of tumor necrosis factor (TNF) receptor superfamily (Johnson et al., 1986), binds to each neurotrophin with similar affinity and also acts as a co-receptor for Trk receptors. Although the exact role of $p75^{NTR}$ in mediating responses to neurotrophins remains less clear,

it has been suggested to enhance binding affinity and influence neurotrophin selectivity of trk receptors as well as to facilitate retrograde axonal signaling (Barker and Shooter, 1994; Hempstead et al., 1991; Miller et al., 1994; Ryden et al., 1995; Lee et al., 1994; Casademunt et al., 1999). More recent data indicate that the $p75^{NTR}$ may have a more important role in neurotrophin-trk signaling that previous thought (Reviewed by Sendtner et al., 2000)

1.3.2. Trk receptor-mediated signaling pathways

Binding to the trk receptors, NTs induce receptor dimerization that results in the phosphorylation of specific tyrosine residues, which creates specific binding sites for intracellular target proteins that bind to the activated receptors via adapter proteins containing phosphotyrosine-binding (PTB) or Src-homology-2 (SH2) motifs. Activation of target proteins can induce several intracellular signaling cascades, including Ras/Raf/MEK/MAPK pathway (Kaplan and Miller, 2000) that induces neuronal differentiation and neurite growth, the phosphatidylinositol 3-kinase (PI3K)/PKB/AKT pathways that mediates the survival functions of the neurotrophins (Crowder and Freeman, 1998) and the association of phospholipase Cγ (PLC-γ) with trk can infleuence intracellular Ca^{2+} levels and protein kinase C (PKC) activity, which seems to play a key role in activity-dependent plasticity (Bibel and Barde, 2000a). It appears unlikely that all three trk receptors trigger identical signaling pathways, and moreover, even the activation of the same trk receptor by different ligands seems to trigger different signaling events (Bibel and Barde, 2000b).

1.3.3. Effects and functions of Neurotrophins

One of the most thoroughly studied properties of NTs is their ability to maintain the viability, by preventing apoptosis, of specific populations of neurons in the nervous system during development (Purves, 1988). It has been demonstrated that NGF has essential roles in maintaining the viability of nociceptive sensory and sympathetic neurons in vivo (Levi-Montalcini, 1987). BDNF was identified and purified on the basis of its ability to prevent the death of sensory neurons not responding to NGF (Barde et al., 1982). In addition, NTs also regulate cell fate decisions, axon growth, dendrite pruning, innervation patterning and the expression of proteins crucial for normal neuronal function, such as, neurotransmitters, ion channels and receptors. In the mature nervous system, NTs continue to play a role in controlling cell phenotype, synaptic function and plasticity, and neuronal regeneration as well as resistance to degenerative stimuli.

1.4. GDNF Family

1.4.1. GDNF family Ligands and their receptors

Another group of neurotrophic facors, which belongs to the transforming growth factor-β (TGF-β) superfamily, is the GDNF family of ligands (GFLs) that comprise glial cell line-derived neurotrophic factor (GDNF) (Lin et al., 1993), neurturin (NTN) (Kotzbauer et al., 1996), artemin (ART) (Baloh et al., 1998b), and persephin (PSP) (Milbrandt et al., 1998). The GFLs signal through a multicomponent receptor system with the transmembrane tyrosine kinase receptor Ret, acting as the common signal transducing component (Durbec et al., 1996; Trupp et al., 1996). Ligand preference and binding of Ret are accomplished by high-affinity binding of the GFLs to a family of glycosyl-phosphatidyl inositol- (GPI-) anchored proteins (GFRα1-4). GFRα1-Ret is the preferred receptor for GDNF, while NTN, ART and PSP preferentially signal through GFRα2-Ret, GFRα3-Ret, and GFRα4-Ret, respectively (Jing et al., 1997; Jing et al., 1996; Treanor et al., 1996; Klein et al., 1997; Baloh et al., 1998a; Enokido et al., 1998), although alternative ligand-receptor interaction also appears to occur, such as GDNF can bind to GFRα2 in the presence of Ret, and NTN and ART can bind to GFRα1 (Baloh et al., 1998b; Jing et al., 1997; Sanicola et al., 1997; Creedon et al., 1997). Besides, evidence has been obtained from c-Ret-deficient cells and sensory neurons isolated from c-Ret knockout mice indicating that GDNF can activate intracellular signaling pathways via GFRα1 independently of c-Ret (Trupp et al., 1999; Poteryaev D, 1999).

1.4.2. GDNF/GFRα1/Ret signaling pathways

In the model proposed by Jing et al (1996), a disulfide-linked dimeric GDNF first binds to either monomeric or dimeric GFRα1 and then the GDNF/GFRα1 complex can interact with Ret and induce its homo-dimerization. Like other receptor tyrosine kinases, Ret has been found to be able to activate various signaling pathways including the Ras-MAPK (Worby et al., 1996; Santoro et al., 1994), the phosphatidylinositol 3-kinase (PI3K)/AKT (Soler et al., 1999; Segouffin-Cariou and Billaud, 2000), Jun N-terminal kinase (JNK) (Chiariello et al., 1998; Xing et al., 1998) and phospholipase Cγ (PLC-γ) (Borrello et al., 1996) dependent pathways. The Ras-MAPK pathways appears to be necessary for the survival and neurite growth stimulated by GDNF and NTN (Creedon et al., 1997; van Weering and Bos, 1997; Xing et al., 1998). Both PI3K/AKT and JNK pathways may also play key roles in mediating the trophic effects of the GDNF family ligands.

1.4.3. Neurotrophic effects of GDNF family

Interest in the GDNF family was initially fuelled largely by the ability of all known members to support the survival of dopaminergic midbrain neurons and spinal as well as facial motor neurons in both in vitro survival and in vivo injury paradigms, considering them as potential therapeutic agents in the treatment of neurodegenerative diseases. GDNF has been identified as a very potent neurotrophic factor for midbrain dopaminergic neurons (Lin et al., 1993), and has also been shown to promote survival and differentiation of sensory, autonomic and motor neurons, by retrograde transport in adult sensory and motor neurons (Trupp et al., 1995; Arenas et al., 1995; Heuckeroth et al., 1998; Henderson et al., 1994). There is a substantial loss of spinal and cranial motor neurons (20-40%), and a corresponding increase of dying cells in GDNF- and GFRα1-deficient mouse embryos compared with wild-type controls (Garces A, 2000 Jul 1; Oppenheim RW, 2000). By contrast, newborn *gdnf*$^{-/-}$ or adult *gdnf*$^{+/-}$ mice show no deficits in unmyelinated innervation in the whisker follicle. NTN and ART, like GDNF, have also many neurotrophic effects; they all support the survival of peripheral sympathetic and sensory neurons as well as midbrain dopamine neurons (Baloh et al., 1998b; Kotzbauer et al., 1996; Horger et al., 1998), while PSP promotes the survival of CNS dopaminergic and motor neurons, but not peripheral neurons (Milbrandt et al., 1998).

1.5. Fibroblast Growth Factors

Fibroblast growth factors (FGFs), a large group of polypeptide growth factors with 23 members (Ornitz and Itoh, 2001), have been characterized by virtue of their mitogenic activities for a variety of cell types of mesodermal and ectodermal origin (Rifkin and Moscatelli, 1989). Fibroblast growth factors constitute a family of trophic factors that regulate diverse processes such as proliferation, migration and differentiation during embryonic development and mediate effects in the adult organisms on maintenance as well as tissue repair. Following binding to the heparan-like glycosaminoglycans of the extracellular matrix (Yayon et al., 1991; Mansukhani et al., 1992; Rapraeger et al., 1991; Klagsbrun and Baird, 1991), FGFs bind to specific receptor tyrosine kinases (FGF receptors, FGFR) and induce receptor dimerization and activation, ultimately resulting in the activation of various signal transduction cascades, such as the PLCγ signaling pathway that is important for mitogenesis and differentiation function of FGFR signaling, Crk-mediated signaling pathway that may propagate a mitogenic signal from FGFR and SNT-1/FRS2 acting as SH2-containing proteins to link FGFR activation to the Ras/MAPK signaling pathway important for growth factor-

induced cell-cycle progression (Lemmon and Schlessinger, 1994; Bellot et al., 1991; Powers et al., 2000). Among members of FGF family, basic fibroblast growth factor (FGF-2) signals through four high-affinity FGF receptors (FGFR1-4) to stimulate mitogenesis and differentiation of neuronal precursors and glial cells in the CNS (Ray et al., 1997). In addition to the presence of FGF-2 and FGFR in glial cells and distinct neuron populations of the CNS, FGF-2 exerts neurotrophic and neurite outgrowth activities in the injured CNS (Grothe and Wewetzer, 1996). In the peripheral nervous system, FGF-2 and its receptors, constitutively expressed in dorsal root ganglia (DRG) and the peripheral nerve (Weise et al., 1993; Weise et al., 1992; Grothe et al., 2001), display an upregulation in DRG and in the proximal and distal nerve stumps following peripheral nerve injury (Ji et al., 1995a; Grothe et al., 1997; Meisinger and Grothe, 1997). It has been found that Schwann cells are the main source of FGF-2 and FGFR mRNAs, and in addition, invading macrophages also strongly express FGF-2 and FGFR1-3 in crushed rat sciatic nerve (Grothe et al., 2001). Exogenously applied FGF-2 mediates rescue effects on injured sensory neurons (Otto et al., 1987) and promotes neurite extension and regeneration (Danielsen et al., 1988; Aebischer et al., 1989). Considering the expression pattern and the effects after exogenous administration of FGF-2, this molecule seems to play a physiological role during nerve regeneration. The neurotrophic activity of FGF-2 on motoneurons can be revealed from the study that when motoneurons from 6-day-old chick embryos are isolated and cultured in the presence of laminin, about 55% of these isolated motoneurons can be maintained with CNTF and the percentage can increase to 96% with CNTF and FGF-2 (Reviewed by Sendtner et al., 2000).

1.6. Neuroregulatory Cytokines

1.6.1. Members and their receptors

Cytokines are a heterogeneous group of polypeptide mediators that have been classically associated with immune activation, inflammatory responses and cell differentiation or death. The neuroregulatory cytokines, consisting of interleukin-6 (IL-6), leukemia inhibitory factor (LIF) (Gearing et al., 1987), oncostain M (OM) (Malik et al., 1989), ciliary neurotrophic factor (CNTF) (Barbin et al., 1984; Lin et al., 1989; Stockli et al., 1989), and cardiotrophin-1 (CT-1) (Pennica et al., 1995a), are structurally and functionally related pleiotropic factors that exhibit overlapping biological avtivities also in the nervous system (Taga, 1996). They exert their diverse biological effects by the formation of stable high affinity oligomeric complexes

with transmembrane receptors, which contain one or more copies of a common transducing receptor subunit, gp130 (Taga, 1996; Kishimoto et al., 1995). Therefore, this group of cytokines is also known as gp130 cytokines. Among them, IL-6 binds to IL-6 receptor (IL-6R), and the IL-6/IL-6R complex then associates with gp130, inducing gp130 homodimer formation to initiate downstream signaling cascades (Murakami et al., 1993). LIF binds to LIF receptor (LIFR), whose structure is similar to that of gp130; LIFR then forms a heterodimer with gp130 (Gearing et al., 1991; Gearing et al., 1992). This type of heterodimer, namely, the gp130/LIFR complex, is also utilized for binding and signaling of other members of the gp130 cytokines, such as CNTF, OM, and CT-1 (Liu et al., 1992; Davis et al., 1993; Pennica et al., 1995b). Although gp130 possess no intrinsic tyrosine kinase domains, it is phosphorylated on tyrosine residues after simulation by the gp130 cytokines (Ip et al., 1992; Taga, 1996). The homo- or heterodimerization of gp130 may trigger the activation of associated cytoplasmic tyrosine kinases that is bound to gp130 (Murakami et al., 1993) and subsequent modification of transcription factors, such as Janus kinase (Jak). In the case of IL-6 signaling, STAT3 and, to a lesser extent and in fewer types of cells, STAT1 becomes activated after cytokine stimulation (Zhon et al., 1994). STAT3 is recruited via its SH2 domain, and tyrosine-phosporylated by juxtaposed Jak kinases (Davis et al., 1993; Darnell et al., 1994; Ihle and Kerr, 1995). Tyrosine-phosphorylated STAT3 proteins then form homodimers via intermolecular SH2-phosphotyrosine interactions and are translocated to the nucleus, resulting in the transcriptional activation of the target genes. Besides, the Ras/MARK signaling pathway is also activated after stimulation of gp130 (Boulton et al., 1994; Satoh et al., 1992). The precise mechanism that links the gp130-dimerization to activation of RAS, including the question of which portion of gp130 is required for this activation remains not fully clarified, but may relate to that tyrosine phosphatase, SHP2, can activiate with gp130.

1.6.2. Neuroregulatory cytokines and neuroprotection

A large body of evidence indicates that neuroregulatory cytokines and their receptors participate in the development of nervous system, regulate oligodendrocyte maturation as well as myelination in the spinal cord and influence axonal growth and neuronal survival. Cells stimulated by gp130 cytokines undergo a wide variety of fates, i.e. growth promotion, growth arrest, differentiation, and specific gene expression. During development of the nervous system, the actions of these cytokines include regulation of neurotransmitter phenotype, differentiation of neuronal precursor cells, survival of differentiated neurons, and regulation

of development of both astrocytes and oligodendrocytes. It is also firmly established that these cytokines are important regulators of the responses of the adult nervous system to neuronal injury, promotion of survival, maintenance of function and regeneration of sensory, motor and sympathetic neurons following injury.

1.6.2.1. CNTF

Originally purified in extracts of chick eye, CNTF was first described as a neurotrophic factor that promote the survival of chick ciliary ganglion neurons in vitro (Nishi and Berg, 1979; Adler et al., 1979; Manthorpe et al., 1986). It is then found by the subsequent cloning of rat, rabbit and human CNTF (Lin et al., 1989; Stockli et al., 1989; Masiakowski et al., 1991) that intact adult peripheral nerve is a rich source of CNTF protein and mRNA., i.e. CNTF is localized inside Schwann cells (Rende et al., 1992). CNTF has been shown to promote neurite outgrowth from NGF-responsive sensory and sympathetic neurons (Barbin et al., 1984) and to support the survival of motor neurons in vitro (Arakawa et al., 1990) and to rescue axotomized facial nerve motor neurons in vivo (Sendtner et al., 1990). In addition, the severe deficits were seen in mice lacking either the CNTFRα or LIFRβ, where deficits of approximately 40% were reported in the facial motor nucleus (Jonakait, 1997).

1.6.2.2. IL-6

Interleukin-6 (IL-6), another member of the neuropoietic cytokines family, was initially characterized in terms of its activities in the immune system and during inflammation (Taga and Kishimoto, 1997). Accumulating evidence also supports a key role of IL-6 in neuronal survival, differentiation, regeneration, and degeneration in the PNS and CNS. There is evidence to show the co-expression of IL-6 and neurotrophins at sites of nerve injury, indicating that neurotrophin-cytokine cross talk is involved in both physiological and pathological processes in the CNS (Otten et al., 2000). An intriguing fact is that in addition to the membrane-bound IL-6R, a soluble form of the IL-6R (sIL-6R) can be generated by shedding of IL-6R or alternatively by splicing of the IL-6R mRNA, which can form a IL-6/sIL-6R/gp130 complex to thereby activate the IL-6 signal transduction pathway (Rose-John and Heinrich, 1994). The neural activities of IL-6 are importantly modulated by sIL-6R. It has been found that cultured sympathetic and sensory neurons as well as PC12 cells require IL-6 and sIL-6R for survival and differentiation (Marz et al., 1999). Besides, the co-administration of IL-6 and sIL-6R was found to delay the progression of motor neuron disease (MND) in

wobbler mice, indicating the neuroprotective mechanism of IL-6 functioning through IL-6/sIL-6/gp130 complex on motor neurons (Ikeda et al., 1996).

1.6.2.3. LIF

Although LIF was initially identified as a glycoprotein that suppressed proliferation of M1 myeloid leukemic cells and induced their differentiation (Tomida et al., 1984), it was afterwards known to be equivalent to cholinergic differentiation factor (CDF) on the basis of its activity in inducing the production of acetylcholine in sympathetic neurons (Yamamori et al., 1989), which is capable of acting on many different tissues at various stages during development and maturity (Yamamori, 1991). LIF was shown to regulate expression of multiple neurotransmitters and neuropeptides in many neuronal cell types (Patterson and Nawa, 1993). In the PNS, LIF was found to promote the neuronal generation from neural crest in vitro and to support the survival of sensory neurons from embryonic DRG (Murphy et al., 1991). Furthermore, it was shown that sciatic nerve axotomy induced a robust increase in expression of LIF in the peripheral nerve in distal segments and the ability of sensory neurons to transport LIF retragradely was enhanced after axotomy, suggesting that LIF, acting as a target-derived neuroregulatory factor, analogous to NGF, play a role in peripheral nerve regeneration (Curtis et al., 1994).

1.7. Pro-inflammatory Cytokines

It is becoming increasingly clear that there exists a bi-directional communication between the nervous and the immune systems. This cross talk mostly depends on cytokines that have functionally been classified as being either pro-inflammatory (Th1-type, stimulatory) or anti-inflammatory (Th2-type, inhibitory) depending on the final balance of their effects on the immune system (Mosmann et al., 1986). Cytokines and other products of the immune cells can modulate the action, differentiation, and survival of neuronal cells, while the neurotransmitter and neuropeptide released from neurons play an important role in influencing immune response. Accordingly, in the nervous system, cytokines function both as immunoregulators and neuromodulators to participate in the complex autonomic, neuroendocrine, metabolic, and behavioral responses to infection, inflammation, trauma, ischemia and other diseases (Sternberg, 1997).

Resident or infiltrating cells can synthesize cytokines in CNS. The major source of pro-inflammatory cytokines in CNS, such as interleukin-1β (IL-1β), tumor necrosis factor-α

(TNF-α) and interferon-γ (IFN-γ), appears to be activated microglia and astrocytes (Giulian et al., 1986; Chung and Benveniste, 1990; De Simone et al., 1998a; Schmidt et al., 1990), however, neurons also produce cytokines (IL-6, TNF-α) (Schobitz et al., 1992). Moreover, following CNS damage or infection, peripheral immune cells, such as macrophages, T cells and neutrophils, can invade CNS due to breakdown of the blood-brain barrier (BBB) and increase local synthesis of pro-inflammatory cytokines as IL-1β, IL-6, TNF-α and IFN-γ. All these molecules functioning as regulators or mediators are instrumental in the course of several PNS neuropathies (Creange et al., 1997).

Since inflammation has been suggested to contribute to the outcome of pathological processes such as stroke, neurodegenerative diseases in nervous system, it is conceivable that pro-inflammatory cytokines, including TNF-α, INF-γ, IL-1β, and aforementioned IL-6, may play important roles also in aging-related sensorimotor disturbances.

2. AIMS OF THE STUDY

The objective of this work was to reveal some of the molecular mechanism underlying the aging-related phenotypic changes of motoneurons and primary sensory neurons during aging. The specific aims were:

To EXAMINE the regulation of neurotrophin-trk signaling in peripheral nerves and target muscles during aging (Paper I)

To STUDY alterations in the expression of GDNF family of ligands and receptors in sensory as well as motoneurons, peripheral nerves and target muscles in senescence (Papers II & III)

To INVESTIGATE alterations during aging in the expression of FGF-2 signaling components and molecules of gp130 family in the somatic motoneurons of the spinal cord and target muscles (Paper IV)

To SEEK evidence of changes in the expression of cytokines in the spinal cord of aged rats, which may provide insights into the actions of cytokines in aging-related sensorimotor dysfunction (Paper V)

3. MATERIAL AND METHOD

3.1. Experimental Animals (Paper I-V)

In this thesis work, a total of 52 young adult (2-3 months old; body weight 200-250g) and 54 aged (30 months old; body weight 240-360g) female and male rats (strain: Bkl, Harlan Sprague-Dawley, Houston, TX) were used. The animals were delivered by a local breeder (B & K, Stockholm, Sweden) at an age of 2 months and were thereafter kept under standardized barrier-breeding conditions with food and water available *ad libitum* (R70 with reduced protein content, Lactamin, Vadstena, Sweden). Under these conditions the median survival time is 30 months (±2 months across cohorts), and herein the 30-month-old rats were defined as aged (see Appendix). Rats showed a progressive deterioration of sensorimotor behaviour during aging, with mild symptoms usually emerging at an age of 24-26 months and being mainly confined to the hindlimbs. All aged rats used in the study disclosed symptoms of behavioral sensorimotor disturbances (stage I-III, see Appendix). All experiments were approved by the Local Ethical Committee (Stockholms Norra Djurförsöksetiska Nämnd; proj. nos. N263/95; N90/97; N54/00).

For tissue collection, the animals were deeply anaesthetized with chloral hydrate (300mg/kg i.p.). For ISH, RT-PCR, Northern blot experiments, the rats were sacrificed by decapitation. The examined tissues were quickly dissected out and divided into segments, which were immediately frozen on dry ice or in liquid nitrogen and stored at -80°C until further processing. For IHC experiments, the deeply anaesthetized rats were perfused transcardially with a solution containing 4% w/v paraformaldehyde and 0.2% picric acid in 0.1M phosphate-buffered saline (PBS). The spinal cords were then quickly dissected out and divided into segments, which were postfixed (1.5hr, 4ºC) in perfusion solution, incubated in 10% Sucrose in PBS at 4°C overnight, washed in PBS, and finally stored at -80°C until further use.

3.2. RT-PCR (Paper I, III. IV, V)

For RNA isolation, the tissue samples from the young adult rats were pooled, whereas those from the aged rats, except for some examined regions were processed individually. Total RNA was isolated from the tissues according to the Trizol protocol (GibcoBRL, Life Technologies, Täby, Sweden). RNA amount and purity was measured in a spectrophotometer (Pharmacia Ultrospec-Plus, Uppsala, Sweden or Eppendorf BioPhotometer, Westbury, USA).

Our detailed RT-PCR procedure has been described in **Paper III**. Briefly, reverse transcription was conducted in a reaction volume of 10µl or 5µl RT-reaction mix (Applied Biosystems, NJ, USA) containing 100ng or 25ng total RNA, incubated at 25ºC for 10 min, 42˚C for 15 min, 99˚C for 5 min, and finally at 5˚C for 5 min in a GeneAmp PCR system 2400 (Applied Biosystems). PCR was carried out by addition of a PCR master mix (Appled Biosystems) to the RT-reaction mix, yielding a reaction volume of 50µl or 25µl. For each experimental sample, three PCR reaction mixtures were prepared and subjected to three different numbers of PCR cycles (e.g. 31, 33 and 35 cycles), which were chosen according to preceding pilot experiments on young adult and aged rat tissues so as to keep the PCR amplification within the range of exponential progression (see Fig. 1). Note that since the

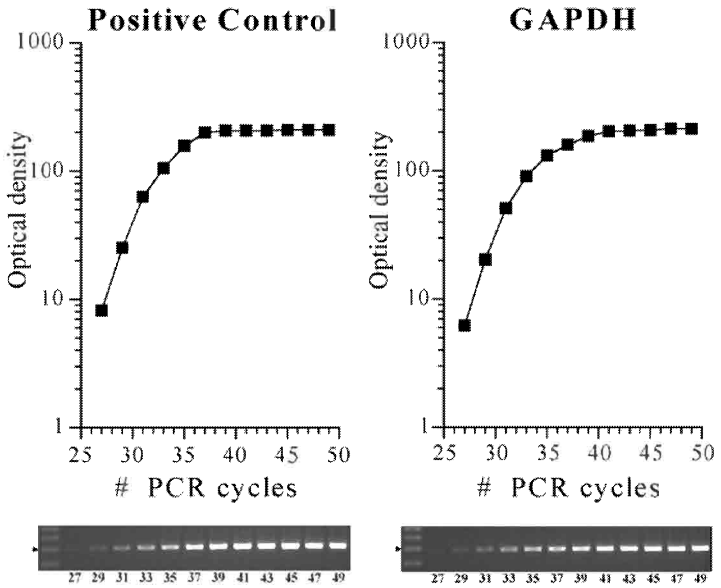

Figure 1. *RT-PCR amplification kinetics for positive control and GAPDH. Optical density values (log_{10} scale), derived from the agarose gels shown below the graphs, were plotted against the number of PCR cycles (27-49). The amount of input RNA for the positive control (pAW109RNA) was 10^4 RNS copies and for GAPDH 0.1ng of spinal cord total RNA. Amplification was linear up to 35 cycles for positive control and GAPDH, and thereafter reached a plateau. The arrowheads indicate the 300bp band in a 100bp DNA ladder, showing bands of appropriate size for positive control (308 bp) and GAPDH (278bp).* ***(Paper III)***

abundance of the examined mRNAs varies, the number of PCR cycles to be used varied accordingly. After 12 min at 95ºC to activate the enzyme, cycling at the chosen number of

cycles was carried out under the following conditions: denaturation at 95°C (15 sec.) and primer annealing/extension at 60°C (30 sec., with an automatic increment of 3 sec/cycle). At the end of the last cycle, the reaction was kept at 72°C for 7 min and then brought to 4°C. In all experiments RT-PCR was performed simultaneously on young adult and aged rat samples, with the internal control (GAPDH or 18S rRNA) run in parallel with the examined mRNAs. The commercial IL-1α primer set DM151/DM152 (Applied Biosystems) was used as a positive control to analyze failures during the PCR process. The oligonucleotide primers used for the PCR reactions were summarized in Table 1. All primer sequences were checked in GenBank to avoid inadvertent homologies. The intron-spanning β-actin primers were used to check for DNA contamination of the samples.

For semi-quantification, 10μl of the PCR reaction product was electrophoretically run on a 1.5% agarose gel containing ethidium bromide (1.25μg/ml). The gels were visualized in a UV-transilluminator and images were captured using an 8-bit CCD camera (Nikon, Japan). Subsequent analysis, using the Optimas 6.5 software (Optimas Co., Bothell, WA, USA), included measurement of the integrated gray levels of each band, correction for local background and normalization against housekeeping mRNA levels. The data, i.e. the mean of the normalized values for the three cycle points, are presented as the percentage differences of the unit ratio between aged and young adult rats. In the evaluation of the results, arbitrary limits were set such that differences of > 100% were considered significant and > 50-100% were considered as tendencies.

3.3. In situ hybridization (Paper II, IV, V)

In situ hybridization (ISH) was conducted as previously described (Dagerlind et al., 1992; Bergman et al., 1996). The oligonucleotide probes (Table 2), evaluated by BLAST, had no significant similarities to other sequences deposited in GenBank. The probes were labeled at the 3'-end with [α-^{35}S] or [α-^{33}P] dATP by using terminal deoxynucleotidyltransferase (Amersham Biosciences) and then purified by use of G-50 micro columns (ProbeQuant™, Amersham Bioscience, NJ, USA). Fourteen μm sections were cut in a cryostat. Following hybridization and stringent rinse, the slide-mounted sections were dipped in NTB2 nuclear track emulsion (Kodak), followed by exposure in the dark at 4° C for 1-4 weeks, yielding a nonsaturated signal with preserved dynamics. After development in Kodak D-19, the sections were counterstained with propidium iodide (Sigma), dehydrated and cover-slipped in glycerol. The hybridization stringency conditions used here do not allow cross-reaction to any related mRNA with less than 90% sequence homology.

Table 1. Primer pairs used in the RT-PCR experiments

Target	Upper primer position	Lower primer position	Product size (bp)	GenBank Accession
NGF	456-480	922-946	491	M36589
BDNF	2296-2318	2762-2786	467	D10938
NT3	308-332	767-791	484	M34643
NT4	367-386	752-774	408	M86742
trk A	1011-1034	1309-1333	323	M85214
trk B	2233-2255	2651-2675	443	M55291
trk C	2377-2400	2598-2622	246	L14447
$p75^{NTR}$	169-192	572-596	428	X05137
18SrRNA	1112-1129	1505-1526	415	M11188
GAPDH	763-787	1017-1040	278	X02231
β-actin	1663-1687	2535-2559	cDNA:433 Genomic DNA:897	V01217
GDNF	32-55	557-581	550 and 472	L15305
NTN	707-731	889-912	206	U78109
GFRα1	667-691	1390-1413	747	U59486
GFRα2	536-560	1091-1113	578	U97143
Ret	2240-2263	2644-2668	429	X67812
FGF-2	612-637	899-923	312	M22427
FGFR1	1194-1216	2049-2072	879	D12498
FGFR2	55-75	421-438	384	L19109
CNTF	278-304	680-705	428	X17457
CNTFRα	686-710	991-1011	326	S54212
LIF	195-220	509-532	338	AB010275
LIFR	2618-2639	2946-2969	352	D86345
IL-1β	293-316	620-639	325	M98820
IL-6	7950-7968	8673-8691	742	M26745
IL-6R	1311-1331	1656-1675	365	NM_017020
gp130	1944-1967	2442-2465	522	M92340
IFN-γ	71-91	376-395	325	AF010466
IFNγR1	762-784	1299-1323	562	AF201901
IFNγR2	1-26	399-423	423	S69336
MHC I	908-932	1217-1241	334	L26224
β_2-MG	25-50	274-293	269	Y00441

To analyse the ISH results, 5-10 slides were systematically sampled for target mRNAs, with each slide containing both young adult and aged cases. In each section, fields were sampled using a Nikon Microphot-FX microscope, equipped with an Ultrapix 1600 CCD camera with a 1536 x 1024 element matrix (Astrocam Ltd., Cambridge, UK). In DRGs, one field per case and section was sampled, covering both the central and peripheral parts of the DRG. In each section of the spinal cord, the left and right motor nuclei were examined and images were captured systematically. Image pairs were generated from each field and presented as a 24-bit RGB image, with the silver grain image and the counter stain image in separate channels. In the recorded images, analyses included silver grains over all DRG neuron profiles with a clearly visible nucleus, and over all neuron profiles in the spinal cord with a diameter exceeding 30μm, thus representing α-motoneurons. A binary threshold was set for the silver grain image, and data was then extracted from the selected cell profiles or area, providing information about their cross-sectional area and the percentage of that area covered with silver grains (labeling density). In total, several decades to several hundred profiles were analysed for each probe and in each age group. Furthermore, in each recorded field the background labeling density was sampled in a representative portion of the interstitium or neuropil. It should be noted that the data analysis of labeling densities was made without any preconceived opinion of cell profiles.

3.4. Northern blot (Paper II)

Total RNAs (30μg/lane) of both aged (individual) and young adult (pooled) spinal cords were separated on a 1% formaldehyde agarose gel and then transferred to Genescreen^{TM} Plus membranes (DuPont NEN, Boston, USA). The RNA was fixed to the membrane by baking at 80^{o}C for 2 hr. Probe labeling has already been described in the section of ISH. The membranes were prehybridized and then hybridized with ^{32}P-labeled GFRα1, Ret and GAPDH probes (see Table 2) at 65^{o}C in 5 x sodium chloride sodium phosphate EDTA (SSPE), 0.5% (w/v) sodium dodecyl sulphate (SDS), 5 x Denhardt's solution and 100μg/ml denatured salmon sperm DNA. The membranes were rinsed to a final stringency of 0.1 x SSPE, 0.1% (w/v) SDS at 55^{o}C and exposed to Fuji Bas UR Imaging plates (Fuji Photo Film Co., Tokyo) for two days. The exposed plates were then read in a Fuji Bas 3000 Bio-imaging Analyzer (Fuji). Each filter was then stripped by boiling and rehybridized according to the following scheme: totally three membranes were used and first hybridized with one of the probes (detecting c-ret, GFRα-1 or GDNF, respectively); e.g. against c-ret mRNA, then

Table 2 Oligonucleotide probes used in the ISH or Northern blot experiments

Target	Length (bp)	Probe position	Probe sequence	GenBank Accession
GFRα1	47	805-851	tggacatgctggtggtgcaggggtgatgtaggccgacctgtacttc	U59486
Ret	50	2527-2576	atctgccaggcgaaggagatgaggtcaccatggtcagtaccctttcatc	X67812
GDNF	50	540-589	cctgcaacatgcctggcctaccttgtcacttgttagccttctacttc	L15305
GAPDH	43	860-902	gtcctcagtgtagcccaggatgccctttagtgggccctcggcc	X02231
FGFR1	40	1511-1543	tcttcacggccactttggtcacgcggttgggttgtgtcctt	D12498
CNTFRα	48	467-514	acagtcacattgaaggtattggggatgtaggtgggggcggacaggtgc	S54212
LIFR	48	3023-3070	catcacggggtcagtgtcctgctcgtcctctgctttggcttgtggctg	D86345
IL-6R	46	501-547	tgcaaacatcacagccttcgtggttggagagggagtgctgcttgga	NM_017020
gp130	48	1908-1955	tcccagcagcgttgtcaggaggaaagctaagcacacaggcacgactat	M92340
IFN-γR	48	80-120	ctccactcccgatctccgcagacagcatcaggaccaccagcagaatca	AF201901
IFN-γR	48	1041-1088	agttcttcctgctctggtgcttctaggctgtctggagttgtcactgtg	AF201901
IFN-γR	48	364-412	cgcataggcagattctctttgtccaaccttggccttaactctggccca	AF201901
MHC I	48	1002-1049	cctttcccacctgtgtttctcttcctcctcctcctcacaacagccacc	L26224
β_2-MG	48	276-323	tgacgtgtttaactctgcaagcatatacatcggtctcggtgggtgtga	Y00441
CGRP	35	664-698	gttccattgagtcacaacattaccatgtccccaga	M11597
GAP43	48	70-117	ctcatcattcttttcaacctgtttggttcttctcatacagcacagcat	M16228

stripped, rehybridized with the GAPDH mRNA detecting probe, stripped again and hybridized for a third time with (in this example) the probe detecting GFRα-1 mRNA. The transcripts visualized by Northern blot with our probes were similar to other independent observations (Reeben et al., 1998).

3.5. Immunohistochemistry (Paper II, IV, V)

The sections of fixed spinal cords were cut at 14 μm in a cryostat, thawed onto gelatin/chrome-alum coated slides. The section-mounted slides were rehydrated in PBS and incubated with polyclonal antibodies at 4ºC overnight or with monoclonal antibodies for 72 hr. All the antibodies were summarized in Table 3. After incubation with the primary antiserum, the sections were rinsed several times in PBS and incubated with fluorescent (FITC or/and Rhodamine Red^{TM}) conjugated secondary antibodies for 30 minutes at 37ºC. Thereafter, the sections were thoroughly rinsed in PBS and mounted in glycerol/PBS (3:1)

containing 0.1% para–phenylenediamine in order to retard fading (Johnson and de C Nogueira Araujo, 1981; Platt and Michael, 1983). In a somewhat modified protocol, sections from the unfixed tissues were transferred to a fixative containing 4% w/v paraformaldehyde and then treated identically as the sections from the perfusion-fixed tissues.

The relevant antibodies have been tested extensively with respect to specificity that can be controlled by preabsorption of the antibody with appropriate antigen, which should abolish staining, or by omission of primary or secondary antibodies, resulting in no detectable labeling, and/or by Western blot demonstrating a single band. Despite these controls, it must be acknowledged that an element of uncertainty still remains as to the specificity of the staining, and consequently antibody labeling was referred to as "-like immunoreactivity (-LI)" or "-immunoreactivity (-IR)".

The tissue sections were examined either in a Nikon Microphot-FX epi-fluorescence microscope equipped with appropriate filter combinations (Nikon, Japan) or a Bio-Rad Radiance Plus laser confocal scanning system (Bio-Rad, Hercules, Ca, USA).

3.6 Statistics (Paper II, IV, V)

One way of analysis of variance (ANOVA) followed by Fisher's LSD was used to test for the differences within or between groups with more than two samples, with the level of significance were indicated as follows: n.s., non significant; *, $p<0.05$; **, $P<0.01$; ***, $p<0.001$ (**Paper II**). Differences between two groups were analyzed by Kruskal-Wallis analysis of variance followed by Mann-Whitney U test, with level of significance $p<0.05$ (**Paper IV, V**).

Table 3 Primary antibodies used in the IHC experiments

Antibody	Raised in	Dilution	Study	Reference
CNTF	Rb	1.500	IV	(Stockli et al., 1991)
ED1	Mo	1.500	IV	(Dijkstra et al., 1985)
ED2	Mo	1.500	IV	(Dijkstra et al., 1985)
FGF-2	Mo	1:400	IV	(Ji et al., 1995b)
GFAP	Mo	1:1000	IV	Sigma
GFAP	Rb	1:1000	IV	(Persson, 1995)
GFRα1	Rb	1:100	III	Dr. Carlos Ibáñez
IL-1β	Go	1:40	IV	R&D systems, UK
IL-6	Go	1:40	IV	(Lemke et al., 1998)
MHC I	Mo	1:400	V	Serotec, UK
OX42	Mo	1:1600	IV	(Robinson et al., 1986)
TGF-β1	Rb	1:80	IV	(Zhu et al., 2000)
TNF-α	Go	1:80	IV	(Acarin et al., 2000)
Transferrin	Rb	1:1000	IV	(Persson, 1995)

4. RESULTS AND DISCUSSION

4.1. Neurotrophin-trk signaling and aging-related sensorimotor impairment (Paper I)

Neurotrophins (NGF, BDNF, NT3, and NT4), signaling through the trk (A/B/C) receptor family alone or together with $p75^{NTR}$, have been suggested to influence cell phenotype, synaptic plasticity, neuronal regeneration and resistance to degeneration in adulthood (Rask and Escandon, 1999). Accumulating evidence indicates disturbed neurotrophin-trk signaling as an underlying mechanism in peripheral neuropathies and aging-related aberrations (Rylett and Williams, 1994). It has been shown from the previous studies that a selective expression of trkA and trkC exists in small and large PNS neurons, respectively, and trkB is expressed by neurons of all size categories in the aged rats (Bergman et al., 1996; Bergman et al., 1999b), and that the expression of trk receptors become downregulated in DRG and spinal motor neurons of aged rats with behavioural sensorimotor deficits (Johnson et al., 1999a; Bergman et al., 1999b). It is interesting to note from lesion experiments and transgenic animals that target-derived neurotrophins can regulate the cognate trk receptor expression in primary sensory neurons both during development and in adulthood (Krekoski et al., 1996; Lindsay, 1992; Raivich et al., 1991; Kitzman et al., 1998). In this respect, trk downregulation in aging DRG and spinal motoneurons may reflect a failure of targets to supply neurotrophic factors. In support of such a hypothesis, the expression of neurotrophins in the peripheral target tissues and peripheral nerves of aged rats was examined by use of RT-PCR.

In target muscles, a significant downregulation of NGF and NT4 mRNAs, as well as tendencies for a downregulation of BDNF and NT3 mRNAs were observed (Fig. 2 A), and notably, the decrease co-varied with the extent of behavioural sensorimotor disturbances among the aged individuals (Fig. 2 B). These regulations have been confirmed in an independent study (Jiang et al., 2002). The amount of extracted total RNA in the individual spinal cord samples was ample enough to allow detection of $p75^{NTR}$ and trk mRNAs by RT-PCR. In line with previous findings (Johnson et al., 1999), tendencies for $p75^{NTR}$ mRNA upregulation (+77%) and trk mRNA downregulation (trkA: -56%, trkB: -27%, trkC: -30%) were observed.

It has been previously inferred that the downregulation of trk receptors may be due to a decreased access to target-derived neurotrophins, suggesting that several of the observed phenotypic changes in aging DRG neurons may be explained by an attenuated neurotrophin-

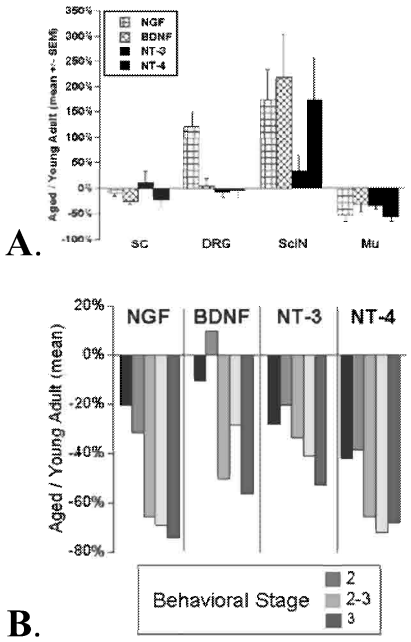

Figure 2. *(A) Relative expression of NGF, BDNF, NT-3 and NT-4 mRNAs in different tissue regions (spinal cord, SC; dorsal root ganglion, DRG; sciatic nerve, SciN; triceps surae muscle, Mu) of aged rats (data from individual cases pooled). (B) Relative expression of NGF, BDNF, NT-3 and NT-4 mRNAs in triceps surae muscles of aged rats (individual). The data of both (A) and (B) are presented as the percentage difference of unit ratio between aged and young adult rats.* ***(Paper I)***

trk signaling (Bergman et al., 1999). The results of **Paper I** lend support to this notion and add evidence that the expression level of trk receptors in adult DRG neurons, at least in part, is regulated by target-derived neurotrophins.

The increased expression of $p75^{NTR}$ mRNA in aged motoneurons may represent a compensatory mechanism for the decreased availability of target-derived neurotrophins in aged rats, by means of a positive effect of $p75^{NTR}$ on formation of high-affinity binding sites, internalisation and retrograde transport of neurotrophins, or possibly by increasing the selectivity of trk receptors for their preferred ligands (Curtis et al., 1995; Bothwell, 1995).

In contrast, the peripheral nerve of aged rats showed a significant upregulation of NGF, BDNF and NT4 but not NT3 mRNAs (Fig. 3 A). This pattern of regulation is analogous to what observed in lesioned nerves of young adult rats, where the upregulation of neurotrophins has been suggested to take place mainly in Schwann cells and to play a role in the regeneration of sensory and motor neurons (Meyer et al., 1992; Funakoshi et al., 1993).

However, the increased levels of neurotrophins in the nerve do not affect the typical phenotypic changes of the axon-lesioned DRG neurons (Verge et al., 1996; Krekoski et al., 1996; Johnson et al., 1995). Conversely, exogenously supplied neurotrophin can partially reverse the 'axon reaction' phenotype of DRG and motoneurons, it seems therefore that exogenously administrated or target-derived, but not Schwann cell-derived neurotrophins can regulate expression of trk receptors in peripheral sensory neurons (Verge et al., 1996; Friedman et al., 1995). The fact that Schwann cells also express neurotrophin receptors in response to nerve lesion in adulthood (Johnson et al., 1988) and during aging (Bergman et al., 2000) may indicate that Schwann cell-derived neurotrophins have a predominantly local effect. At the site of nerve insult, neurotrophins may exert effects in glial cell reaction and may also be important for attracting the growth cones of the regenerating axons without affecting other phenotypic switches in the 'axon reaction' of the neurons (Verge et al., 1996; Krekoski et al., 1996).

In conclusion, the results of **Paper I** add evidence suggesting that there is an aging-related attenuation of neurotrophin signaling between target tissues on one hand and DRG and motoneurons on the other hand. Furthermore, that target-derived neurotrophins seem to regulate the expression levels of trk receptor mRNAs in both DRG and motoneurons also during aging (c.f. Griesbeck et al., 1995).

4.2. Possible protective effect of GDNF signaling on aged sensory and motor neurons (Paper II and III)

Primary sensory neuron (PSN) and spinal motoneurons show a complex pattern of changes at the cellular level during aging. It is well established that aging is associated with axon aberrations, such as axon atrophy and dystrophy, de- and dysmyelination, and signs of degeneration in peripheral nerves and spinal roots (Knox et al., 1989; Fujisawa, 1988; Bergman and Ulfhake, 2002). It has been shown that the attenuation of sensory innervation more extensively involves terminal branches of myelinated proprioceptive/exteroceptive fibres than unmyelinated nociceptive fibres, thereby, aging individuals are more affected by disturbed propriception that by changes in nociception (Woollacott, 1986; Bergman and Ulfhake, 2002). In addition to the aforementioned neurotrophin signaling, the more successful aging of nociceptive than of proprioceptive primary sensory neurons may be due to other neurotrophic factors in adulthood. GDNF family ligands (GDNF, NTN, PSP and ART) belong to such candidates owing to the fact that GDNF was found to be a powerful trophic substance for PSNs (Moore et al., 1996; Bennett et al., 1998a; Matheson et al., 1997; Buj-

Bello et al., 1995) and motoneurons (Oppenheim et al., 1995; Yan et al., 1995; Henderson et al., 1994; Vejsada et al., 1998; Sanchez et al., 1996; Li et al., 1995). Moreover, evidence indicates that GDNF can protect the B4-binding nociceptive neurons as well as the axon conduction velocity of C-fibers in axon-lesioned adult sensory neurons (Bennett et al., 1998b). The main signaling pathway for GDNF is through binding to GDNF receptor GFRα1; the GDNF/GFRα1 complex binds and activates the c-Ret receptor that is the signal-transducing component. Both GFRα1 and c-Ret are expressed in adult motoneurons as well as in subpopulations of primary sensory neurons (Nakamura et al., 1996; Klein et al., 1997; Colucci-D'Amato et al., 1996; Trupp et al., 1997; Buj-Bello et al., 1995).

By use of *in situ* hybridisation (ISH) and immunohistochemistry (IHC), it was shown that the expression of GFRα1 and c-Ret was upregulated in spinal motoneurons and DRG neurons (Paper II). Based on the observations made in the developing kidney, enteric neurons, and the nigrostriatal pathways (Schaar et al., 1993), both GFRα1 and c-Ret mRNA appear to be regulated by the amount of accessible ligands, i.e. GDNF (and possibly also NTN). In the mature rat, insults of the peripheral motor axon result not only in an upregulation of GDNF both in the target muscles and the supporting Schwann cells, but also in an increase of GFRα1 mRNA in the latter (Naveilhan et al., 1997; Trupp et al., 1997). Accordingly, there is an upregulation of both GFRα1 and c-RET in the axotomized motoneurons, while c-Ret continues to be expressed at only low levels in the nerve and target muscle (Naveilhan et al., 1997). GDNF synthesized in Schwann cells and target muscles may act as a ligand to the GFRα1/c-Ret receptor complex, expressed by the lesioned motoneurons, but also as a soluble GDNF/GFRα1 complex that could signal through the c-Ret receptors (Yu et al., 1998). The upregualtion of GFRα1 and c-Ret in the aged sensory and motor neurons seems to indicate an increased expression of GDNF. However, the expression of GDNF mRNA was below detection level in the spinal cords of both young adult and aged rats with ISH and Northern blot, suggesting that an increased level of the ligand may derive from Schwann cells of the peripheral nerve and/or from the target muscles.

The expression of GDNF and NTN mRNA was examined by the more sensitive RT-PCR in aged dorsal root ganglia, spinal cord, peripheral nerve as well as target muscles (Paper III). It was then found that GDNF, but not NTN, was strongly upregulated in target muscles and to a lesser extent also in peripheral nerve, roots and DRGs, but not in the spinal cord of the aged rats (Fig. 3)

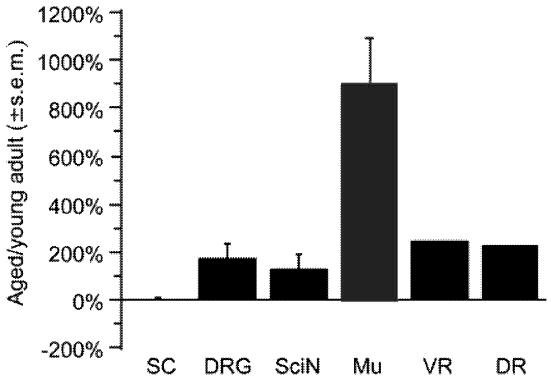

Figure 3. *Relative expression of GDNF mRNA in different tissue regions (spinal cord, SC; dorsal root ganglion, DRG; sciatic nerve, SciN; triceps surae muscle, Mu; ventral root, VR; dorsal root, DR) of aged rats. The mean gray values obtained for GDNF, run at three different PCR cycle points, were corrected for local background and normalized against GAPDH mRNA levels. The data, i.e. the mean of the normalized values for the three cycle points, are presented as a ratio between aged (data from individual cases pooled) and young adult rats. Note that the aged rats were analyzed individually, except for the ventral and dorsal roots. (Paper III)*

In the adult, GDNF is constitutively expressed in neurons and astrocytes of the CNS, in Schwann cells and satellite cells, in skin and, at least in some species, in skeletal muscle (Trupp et al., 1995; Hammarberg et al., 1996; Schaar et al., 1993; Trupp et al., 1997). The increase of GDNF mRNA was dramatic in the muscles, while the changes in the peripheral nerve, roots and DRG were more discrete. Thus, the main source of GDNF during aging appears to be the target muscles. Albeit being sensitive, the RT-PCR protocal used here cannot determine the cellular origin of the mRNA species. For example, it is possible that terminal Schwann cells rather than muscle fiber, or both, contribute to the increased amounts of GDNF mRNA in the muscle samples seen in this study. Both GFRα1 and GFRα2 were upregulated in aged DRG. GFRα2 is the preferred receptor of NTN, but we could not detect any significant changes in NTN mRNA during aging. Since GDNF also binds to GFRα2 (Bennett et al., 1998a), it does not necessarily reflect a difference in GDNF/NTN dependence on GFRα2: GDNF, being perhaps the most potent neurotrophic factor for motoneurons discovered so far, is retrogradely transported and can rescue motoneurons fro axotomy-induced cell death, improve motoneurons regeneration and has been claimed to preserve the cholinergic phenotype of lesioned motoneurons (Naveilhan et al., 1997; Yan et al., 1995; Henderson et al., 1994; Trupp et al., 1997). The increased GDNF signaling between the target muscles and motoneurons may serve as a compensatory mechanism in the context of

decreased neurotrophin-trk signaling in aging motoneurons, promoting axon regeneration (Naveilhan et al., 1997; Trupp et al., 1997), sprouting, and muscle fibre re-innervation (Nguyen, 1998), and may be mechanistic in the protection of cell-body size and cholinergic phenotype (Johnson et al., 1999a). It has been found that GDNF has inhibitory effects on the generation of radical oxygen species (ROS) (Irie and Hirabayashi, 1999) and may have protective function on motoneurons (Irie and Hirabayashi, 1999). For the DRG neurons affected by aging, increased GDNF-GFRα1(α2)/Ret signaling in aged sensory pathways may explain the preserved B4 phenotype and the lack of cell body atrophy among the nociceptive sensory neurons, while the large/RT97-immunopositive propriceptive sensory neurons show signs of both cell body atrophy and extensive axon degeneration in senescence (Bergman and Ulfhake, 1998; Fundin et al., 1997).

As summarized in Fig. 4, the regulatory changes with an increased GDNF signaling observed in aged rats in a milieu of decreased neurotrophin-trk signaling seem consistent with the phenotypic changes characterizing senescence.

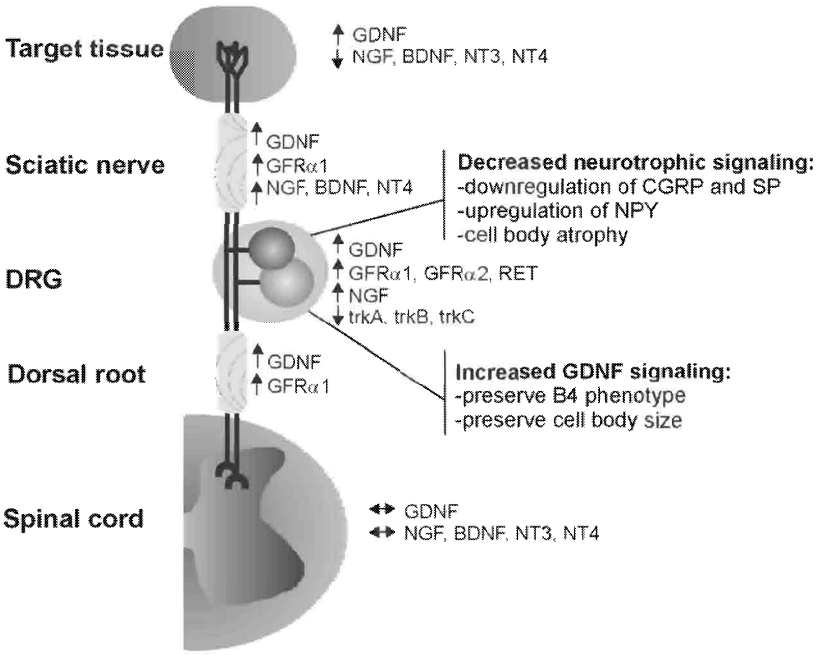

Figure 4. *Schematic representation summarizing expression changes observed in aged rats. The changes occurred are indicated by arrows as follows: up-regulation (↑), down-regulation (↓), no change (↔). (Modified from* ***Paper III****)*

4.3. Alterations in the expression of FGF-2 signaling components and gp130 cytokines in senescent motoneurons and target muscles (Paper IV)

Aging motoneurons show a distinct pattern of phenotypic changes with small loss of motoneurons, a preserved cholinergic phenotype and no signs of cell body atrophy (Johnson et al., 1995). In addition, both CGRP and GAP43 are marked upregulated in aged motoneurons (Johnson et al., 1995). Our previous data of a decreased neurotrophin (BDNF, NT3, NT4)-trk signaling and an increased signaling of GDNF-GFRα1/c-Ret during aging were proposed to be responsible for the 'aged' motoneurons phenotype. Besides, members of the fibroblast growth factor (FGF) family e.g. FGF-2 as well as the neuroregulatory cytokines signaling via gp130, which exert effects on motoneurons during development and also act as 'survival' signals in traumatic and neurodegenerative conditions, may be of importance in senescence. Thus, we examined if these signaling pathways are regulated in spinal motoneurons and target muscles during aging by RT-PCR, ISH and ICH techniques. While we could establish a small upregulation of FGF receptor 1 (FGFR1) in motoneurons and a tendency for increased FGF-2 labeling in spinal cord astroglia, no regulatory change in gp130 signaling components (CNTF, IL-6, LIF, CNTFRα, LIFR, IL.6R or gp130) could be observed in the spinal cord during aging. However, in the target muscles there was a marked downregulation of IL-6 and FGF-2 mRNAs, and an upregulation of FGFR1, CNTFRα and gp130 mRNAs in the target muscles during aging. FGF-2 as well as IL-6, LIF and CNTF have been shown to be 'survival' signals for motoneurons. The signaling through gp130, by way of LIFR, IL-6R and CNTFRα are important during prenatal and postnatal development. Evidence has also been provided that at least some of these ligands are retrogradely transported, and thus they may act as a target-derived survival factor as well as by way of paracrine/autocrine loops. Therefore, it is conceivable that maintained signaling of the gp130 cytokines and the possible increased FGF-2/FGFR1 signaling are involved in the phenotypic makeup of in aged motoneurons. Given the analysis result of FGFR1 expression, the likely sources of FGF-2 for the motoneurons are astroglia in the spinal cord and Schwann cells of the peripheral nerve compartment.

The increased levels of FGFR1, gp130, CNTFRα and IL-6R in the target muscle may reflect the repair situation in senescent muscles.

4.4. Role of cytokines in the aged spinal cord characterized by gliosis associated with axon aberrations and disturbed myelination (Paper V)

The normal function and homeostasis of the central nervous system (CNS) is dependent on an intricate network of cross talk between neurons and glia, which have been found to play an important role in injury and neurodegenerative processes in the CNS. With increasing age, the ratio of glial cells to neurons increases resulting in gliosis, the extent of which correlates with loss of synaptic bouton, axon aberrations, disturbed myelination and behavioural sensorimotor impairment (Kullberg et al., 1998; Kullberg et al., 2001). Albeit these changes are extensive during aging, the underlying molecular cross talks are not well understood. Cytokines, being multifunctional pleiotropic proteins that play crucial roles in cell-to-cell communication and cellular activation, might be good candidates to be investigated in this respect. Herein, we examined the expression of several cytokines (INF-γ, TNF-α, IL-1β, IL-6, TGF-β1) in different glial cell types as well as spinal cord motoneurons of aged rats and young controls by use of ISH, IHC and RT-PCR.

The key findings was a significant increase of INF-γ mRNA in the aged spinal cord, concomitant with the upregulation of INF-γ receptor complements (α-chain, INF-γR1; β-chain, INF-γR2) and MHC I as well as β_2-MG, known to be induced by INF-γ. There is evidence that in CNS both astroglia and microglia can synthesize IFN-γ (De Simone et al., 1998b), and neurons as well as glia express the IFN-γ receptor (IFN-γR), which is a receptor complex composed of a ligand binding subunit (α-chain) (Aguet et al., 1988) and a transmembrane factor (β-chain) essential for downstream signaling (Hemmi et al., 1994; Tau and Rothman, 1999). It was found here that motoneurons and probably also glia-like cells express increased levels of IFN-γR mRNA during aging, and it seems as if the upregulation of β-chain is more marked than that of α-chain, based on the RT-PCR results. IFN-γ receptor signaling upregulates MHC antigen expression, which under normal conditions is very low in CNS. The increased levels of both MHC I and β_2-MG, in neurons and glial populations overlapping those with increased levels of IFN-γR, may further provide evidence for an increased IFN-γ signaling in senescence. The increased expression of IFN-γ observed here agrees with the results from aged mice brain (Wei et al., 2000). Signs of increased IFN-γ signaling in aged rats were also evident in glia-like cell profiles in white matter region affected by axon dystrophy and disturbed myelination, as well as in spinal root. In such regions, both astroglia and activated microglia increase to maintain the organization of white matter, to clear myelin debris and to pave way for subsequent remyelination after demyelination damage (Avellino et al., 1995). In this respect, through paracrine or autocrine loops IFN-γ signaling may serve to activate resident astroglia and microglia in response to aging-related myelin breakdown (Franklin and Hinks, 1999). Besides, there is mounting

evidence showing that IFN-γ may be involved in the concerted processes of neuronal differentiation (Improta et al., 1988), neuronal protection from apoptosis (Chang et al., 1990), signal transmission (Vikman et al., 2001), motoneuron response to axon lesion (Linda et al., 1998), and synaptic plasticity during development (Corriveau et al., 1998). Thus, increased IFN-γ signaling in aged spinal cord implies that IFN-γ may play an important role in the process of demyelination-association inflammation and changes in synaptic connectivity.

In addition, TNF-α IR was observed in both micro- and astroglial cells, but found no change in senescence. Increased levels of IL-1β, IL-6 and TGF-β1 IRs were seen in aged astroglial cells, and TGF-β1 IR was also increased in aged microglia. An increased expression level of TGF-β1 mRNA during aging was seen with RT-PCR. Neuronal survival is critically dependent on glial function, which can exert both neuroprotective and neurotoxic influence (Giulian et al., 1993). Glial cells are a primary target of cytokines and are activated in response to many cytokines, including TNF-α, IL-1β and IL-6. This activation can trigger further release of cytokines that might enhance or suppress local inflammatory responses and neuronal survival. Generally, immune cells, such as macrophages, T cells and neutrophils, which can invade the CNS after injury or inflammation, are a rich source of cytokines. Likely, resident neuronal and glial cells might produce cytokines within CNS. Because TGF-β1 exert profound effects in oligodendroglia cell recruitment, oligodendroglia maturation, Schwann cell growth as well as activity promotion and myelin formation (Franklin and Hinks, 1999), the fact that small philopodia-carrying cellular profiles around the central canal were strongly labeled with TGF-β1 in aged rats may represent newly formed cells recruited for glial cell differentiation. Besides, TGF-β1 increases GFAP expression, the main intermediate filament protein of astrocytes; it seems reasonable that increased levels of TGF-β1 may contribute to the upregulation of GFAP in senescence. Our data also indicated the upregulation of IL-1β in astroglia during aging, which might be in line with the notion that it promotes astrogliosis in concert with TGF-β1 (Laping et al., 1994). IL-1β can upregulate IL-6 as well, and we observed increased IL-6 IR in aged astroglia. In contrast, IL-6 mRNA was unchanged in the aged spinal cord. Based on the evidence that IL-6 can cross the blood brain barrier (Gadient and Otten, 1997), this discrepancy may have its explanation in take-up of systemic IL-6 that has been found to increase in serum from aged mice and humans (Dobbs et al., 1999; Spaulding et al., 1997; Perry and Brown, 1992; Ridley et al., 1989). Overall, cytokines, as important immunoregulators and modulators of neural functions and neuronal survival, appear to be involved in controlling various events in phenotypic changes during aging.

5. GENERAL DISCUSSION

5.1 The scenario of senescent sensorimotor impairments

It has been commonly accepted that aging is associated with loss of cognitive powers and motor function. The dictionary definition of *senile* as "showing characteristics of old age; weak of mind and body" mirrors this fact. Impaired mobility and falls are, after cognitive disorders, the major cause of diminished quality of life and lost self-reliance in older persons. The prevalence of mobility dysfunction, which is 14% of the elderly population from age 65 to 74 years, increases sharply thereafter, involving almost half of those older than 85 years (Odenheimer et al., 1994). Certain neurological symptoms and signs include muscle weakness, increased proprioceptive thresholds and gait disorders characterized by small steps, shuffling, hesitation, poor balance as well as unstable turn, which are often regarded as part of "normal aging". Similar ranges of behavioural deficits are encountered in aged rodents that may possibly serve as a useful model (see Appendix) in trying to elucidate the mechanisms underlying these disturbances. Intriguingly, both humans and rodents exhibit a considerable variability in the effects of aging, which means that some individuals exhibit extensive alterations with age, whereas others show little or no signs (Lamberts et al., 1997). The fact that it seems possible to distinguish between "unsuccessful" and "successful" patterns of aging in several species may indeed facilitate to identify factors of potential importance in the generation of functional deficits during aging. The pattern of changes seen in "senile muscle atrophy", and variations in gait as well as balance control correlate with increased thresholds for exteroceptive and proprioceptive sensations in aged animals and humans which have been considered to be induced by loss of innervation (Kokmen et al., 1977; Larsson, 1982; Dyck et al., 1984; Woollacott, 1986; de Neeling et al., 1994), neuron atrophy in certain cell populations, loss of neuronal connections, axon dystrophy, myelin aberrations, as well as characteristic phenotypic changes in gene-expression pattern. In addition, target failure or a breakdown in the nerve-target interaction is probably a critical event in the aging process of sensory and motoneurons.

5.2 Regulation of neurotrophic signaling in senescent sensory and motoneurons as well as target muscles

Aging sensory neurons show a complex pattern of changes at cellular level, with a small unselective loss of neurons, selective cell-body atrophy, abundant axon aberrations, and phenotypic alterations in the expression pattern of neurotrophic receptors (Bergman et al.,

1996; Bergman et al., 2000; Bergman et al., 1999a; Bergman et al., 1999b; Bergman and Ulfhake, 1998; Fundin et al., 1997). Evidently, the level of trk expression in aged sensory neurons is downregulated, concomitant with downregulation of neurotrophin mRNAs in target muscles, which may reflect a failure of targets to synthesize neurotrophic factors. Besides, the decrease of neurotrophin mRNAs in aged muscle co-varies with the extent of behavioural sensorimotor disturbances among individuals. All these findings imply an attenuated neurotrophin signalling between target tissues and PSN in senescence. Conversely, in the peripheral nerve there is a significant upregulation of all neurotrophins mRNAs except NT3 during aging, suggesting that neurotrophins produced by Schwann cells may play a local role in the regenerative processes of sensory neurons (Meier et al., 1999; Anton et al., 1994), since it seems that target-derived, but not Schwann cell-derived, neurotrophins exert effects on the regulation of trk-receptor expressions in PSN. In contrast to trk receptors, the expression of $p75^{NTR}$ mRNA is slightly increased in aged PSN (Bergman et al., 1999b; see also **Paper I**). The increased ratio of $p75^{NTR}$ to trk might represent a compensatory mechanism for the decreased availability of target-derived neurotrophins in aged rats, by means of a positive effects on formation of high-affinity binding site, internalisation and retrograde transport of neurotrophins, or possibly by increasing the selectivity of trk receptors for their preferred ligands (Curtis et al., 1995; Bothwell, 1995). A number of studies have shown that $p75^{NTR}$ may signal independently of trk receptors, possibly mediating responses ranging from apoptosis to cell migration and differentiation (Verdi et al., 1994; Casaccia-Bonnefil et al., 1996; Carter and Lewin, 1997; Frade et al., 1996; Anton et al., 1994; Rabizadeh et al., 1993; Bredesen and Rabizadeh, 1997). Although $p75^{NTR}$ may activate cell-death pathways in PSN under certain circumstances, the net effect in vivo of $p75^{NTR}$ expression appears to be promotion of survival in PSN. Besides, in aged rats there is an increased GDNF-GFRα1/Ret signaling during aging (see **Paper II, III**) that is of considerable interest because GDNF can protect small nociceptive PSN, but has a negligible effect on large myelinated fibres following axon lesioning (Bennett et al., 1998a; Molliver et al., 1997; Munson and McMahon, 1997), which may add evidence on why myelinated are more affected than unmyelinated sensory neurons.

Aging motoneurons show a distinct pattern of phenotypic changes, as well as extensive signs of axon aberrations that may result in disconnection to an intact set of target muscle cells. A successive dropout of motoneurons has been suggested to be responsible for the changes seen in aging skeletal muscle. Motoneurons still connected to the muscle may try to compensate through collateral re-innervation (Larsson, 1995; Larsson, 1982; Edstrom and

Larsson, 1987), a process where terminal Schwann cells may play a key role. It has been shown that aged motoneurons appear to have a preserved capacity of growth and regeneration to reinnervate target muscle fibres following axon lesions (Kawabuchi et al., 1995; Kanda and Hashizume, 1991). Thus, the process underlying senile muscle atrophy is more complex than just a dropout of parent motoneurons, if senile muscle atrophy would be of neurogenic origin. It is conceivable that number of muscle fibres may decrease during aging due to a restrained regenerative capacity of the muscle tissue per se. In aged rats, skeletal muscles show downregulation of all neurotrophin mRNAs and the degree of decrease correlates with the extent of behavioural sensorimotor impairment among the individuals (**Paper I**), confirmed by Jiang et al., 2002. Combined with the evidence of downregulation of trkB and trkC mRNAs in aged motoneurons (Johnson et al., 1996; Johnson et al., 1999b), the pattern of regulation of neurotrophin-trk signaling in senescence suggests that spinal motoneurons compete for a decreasing amount of target-derived trk ligands. The loss of muscle fibres and the atrophy of the remaining fibres will mutually impose a reduced target size where the remaining muscle fibres express neurotrophins at a decreased level. In contrast to trk, $p75^{NTR}$ is markedly upregulated in aged motoneurons, which may work as an enhancer of neurotrophin-trk signaling in a situation of diminished access to target-derived neurotrophins. Moreover, an increased GDNF signaling between the target muscles and the motoneurons may serve as a compensatory mechanism in the context of decreased neurotrophin-trk signaling in aging motoneurons, promoting axon regeneration (Trupp et al., 1997), sprouting, and muscle fibre re-innervation (Nguyen, 1998); and may be mechanistic in the protection of cell-body size and the cholinergic phenotype. It remains to be determined if the cellular source of GDNF is the muscle fibres and the terminal Schwann cells located in the muscle tissue.

In addition to the NGF and GDNF families, members of FGF family as well as the neuroregulatory cytokines signaling via gp130 are the important molecules with neurotrophic activities for spinal motoneurons and target muscles. FGF-2 as well as IL-6, LIF and CNTF have been shown to be survival signals for motoneurons. The signaling through gp130, by way of LIFR, IL-6R and CNTFRα are important during prenatal and postnatal development. The results of a slight upregulation of FGF-2/FGFR1 signaling and maintained expression levels for gp130 signaling components indicate that both these families may play a role in sculpting phenotype of the aged motoneurons. Profound changes were seen in the regulation of these pathways in the target tissues, indicating a role in the regeneration process of aged muscle.

Table 4 Summary of changes in neurotrophic signaling molecules in aged motoneurons and target muscles

Ligand	Muscle	SciN	SC	Receptor	MN
BDNF	↓	↑	NC	trkB**	↓
NT3	↓	NC	NC	trkC**	↓
NT4	↓	↑	NC	trkB**	↓
				$p75^{NTR}$**	↑
GDNF	↑	↑	NC	GFRα1/Ret	↑
NTN	NC	NC	NC		
FGF-2	↓	NC	NC	FGFR1	↑
CNTF	NC	NC	NC	CNTFRα/gp130	NC
IL-6	↓	Low	NC	IL-6R/gp130	ND/NC
LIF	NC	↑	NC	LIFR/gp130	NC
IGF*	↑		NC	IGFR*	NC

↓, downregulation; ↑, upregulation; NC, no change; ND, not detect; Low, low expression; SciN, sciatic nerve; SC, spinal cord; MN, motoneuron
*, Edstrom, et al., in preparation; **, Johnson, et al, 1995, 1999.

5.3 Possible detrimental or beneficial actions of cytokines in the senescent spinal cord

Cytokines are involved, detrimentally and/or beneficially, in controlling neuronal and glial activation, proliferation, differentiation and survival, thus influencing development degeneration and regeneration of nervous system. In aged spinal cord marked by gliosis with axon dystrophy and de-/dysmyelination, there is an increased IFN-r signaling with a downstream upregulation of MHC I and β_2-MG, suggesting that the increase of IFN-γ may be of relevance not only for the glial reaction to the aging-related axon aberrations but also the loss of synaptic connection in senescence. IFN-γ, a predominantly proinflammatory cytokines, exerts profound effects on various cell types of CNS including, astroglia, microglia, oligodendrocytes, and neurons. There is mounting evidence showing that IFN-γ may be involved in the concerted processes of neuronal differentiation (Improta et al., 1988), neuronal protection from apoptosis (Chang et al., 1990), signal transmission (Vikman et al., 2001), motoneuron response to axon lesion (Linda et al., 1998), and synaptic plasticity during development (Corriveau et al., 1998). It has also been suggested that IFN-γ promotes cholinergic differentiation of motoneurons (Erkman L, 1989). Therefore, increased IFN-γ

signaling in aged spinal cord may contribute to the process of inflammatory responses characterized by gliosis, axon aberrations as well as disturbed myelination and the promotion of regeneration and survival of neurons during aging.

IL-1β, capable of neurotrophic and neurotoxic actions, is thought to be central to CNS inflammatory modulation in response to CNS injury or systemic insult. Increased levels of IL-1β in astroglia during aging would be consistent with the notion that it promotes astrogliosis in concert with TGF-β1 (Laping et al., 1994). TGF-β1 stimulates astrocyte expression of IL-6 and synergizes with IL-1β and TNF-α for enhanced IL-6 production (Jin and Howe, 1997). IL-6 can exert opposite actions on neurons, triggering either neuronal survival after injury or causing neuronal degeneration. In this respect, IL-6 has been proposed as a developmental neurotrophic factor (Gadient RA, 1994; Wagner JA., 1996), but also a neurodegenerative factor (Mizuno T, 1994). In most pathological conditions, the effect of TGF-β1 seems to be beneficial on repair process (Finch et al., 1993; Flanders et al., 1998). However, overexpression of TGF-β1 within the CNS has a detrimental effect.

To sum up, it is likely that the contribution of cytokines to inflammatory responses of senescent spinal cord does not involve a single mechanism on one specific cell type, but rather depends on several actions on a variety of cell types, which might be detrimental or beneficial depending on the cellular milieu. The functional outcome of cytokines will be determined by the concentration of the particular mediator, level of receptor expression on the responding cell, levels of soluble receptors present that can function as either agonists or antagonists, and the presence of other cytokines that can modulate the functional response.

5.4 Conclusions and future directions

The picture of neurotrophic regulation and molecular mechanism during aging is still far from clear, in need of more evidence to fill many gaps in our understanding. From the work presented above, it might seem that changes in neurotrophic signaling may trigger phenotypic changes occurring in senescent sensory and motoneuron as well as target tissues. Likewise, growth factors and cytokines are also involved in many aspects of this process. The elucidation of the downstream signal transduction pathways of neurotrophic factors and other molecules in senescent cells is an important future step, since it might lay the basis for the development of interventions that may retard the intrinsic rate of aging and the cluster of phenotypic changes of the senescent state. Certainly, suffice it to say that much remains to be deciphered about the molecular mechanisms behind senescent sensorimotor impairments.

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