NEUROTROPHIC FACTORS IN SPINAL PAIN TRANSMISSION

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Chapter 1

Introduction

This thesis describes a series of experiments on the role of neurotrophic factors in spinal pain transmission. The details of these studies are found in the Chapters 2-5. As an introduction to these studies a general overview will be given of the organization of the pain system and the involvement of neurotrophic factors in sensory transmission, followed by a short description of the aim of this thesis.

ORGANIZATION OF THE PAIN SYSTEM

According to the definition of the International Association for the Study of Pain (IASP), pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. This definition implies that in order to perceive a stimulus as painful, both the sensory (Fig. 1) and the affective-motivational (Fig. 2) components of pain have to be experienced. In other words nociception or the detection of tissue damage is not the same as pain, because pain includes not only the detection of tissue damage but also the conscious perception of this damage as an unpleasant experience. Much progress in our understanding of the affective-motivational component of pain has been made in recent years (Tracey, 2005), especially by means of functional imaging studies, but a detailed description of these studies is beyond the scope of this introduction. Yet most of our knowledge regarding the organization of the pain system relates to the nociceptive component of pain and this is also the main subject of this thesis, with a focus on the spinal cord.

Pain and temperature are perceived by a system, which is anatomically and functionally distinct from the mechanosensory system, the latter being responsible for the perception of mechanical stimuli, e.g. discriminative touch, vibration, pressure, cutaneous tension, joint position and many other types of proprioceptive information. In virtually all tissues of the body, with the notable exception of the central nervous system, the sensation of pain is initiated by various types of nerve endings collectively termed nociceptors. Nociceptors appear as bare, relatively unspecialized nerve cell endings. They are able to transduce a variety of (potentially) damaging stimuli (including heat, cold, mechanical and chemical inputs), into receptor potentials which may then trigger action potentials. The cell bodies of nociceptors are located in dorsal root ganglia (DRG) or in the trigeminal ganglion and give rise to one axonal process which divides into two branches, one projecting to the periphery and one projecting centrally into the spinal cord or brainstem. There are two major subclasses of nociceptors: Aδ and C nociceptors. Aδ nociceptors are characterized by their thinly myelinated fibers and respond either to potentially damaging mechanical (Aδ mechanosensitive nociceptors) or to mechanothermal (Aδ mechanothermal nociceptors) stimuli. C nociceptors on the other hand are characterized by unmyelinated fibers that often respond to mechanical, thermal as well as chemical nociceptive stimuli and are therefore called polymodal nociceptors. Since Aδ fibers are myelinated, they convey action potentials faster than C fibers. In

human volunteers, by selectively blocking Aδ fibers using a pressure cuff, it was found that Aδ fibers are responsible for the so called first pain, a sharp and short lasting pain, while C fibers are responsible for the second pain, a delayed, diffuse type of pain, often outlasting the duration of the stimulus. C nociceptors are further subdivided into a peptidergic and a non-peptidergic subgroup of C nociceptors (Snider and McMahon, 1998) (Fig. 3). These two groups are further characterized by expressing different neurotrophic receptors and show different central termination areas.

Peptidergic nociceptors, in addition to the classical neurotransmitter glutamate, contain one or more neuropeptides such as Substance P (SubP), Calcitonin Gene-Related Peptide (CGRP), galanin, somatostatin and others. They also express TrkA, the receptor for Nerve Growth Factor (NGF) (Michael et al., 1997), and use Brain-Derived Neurotrophic Factor (BDNF) as a transmitter (see later). Non-peptidergic nociceptive neurons only use glutamate as transmitter. They are further characterized by expression of the plant lectin isolectin B4 (IB4) and contain the receptor components for Glial cell line-Derived Neurotrophic Factor, i.e. RET and GFRα-1, which are also discussed later (Molliver et al., 1997).

In the spinal cord, the peptidergic nociceptors terminate in lamina I and the outer layer of lamina II (IIouter), the most superficial layers of the spinal dorsal horn according to (Rexed, 1952). Here they synaptically contact various types of neurons, including nociceptive specific neurons in lamina I that project to the brainstem and thalamus. Many of these neurons express Neurokinin-1 (NK-1), the receptor for SubP. It has been shown (Mantyh et al., 1997) that following selective ablation of these NK-1 neurons by a toxin, saporin, that was conjugated to SubP, baseline thermal and mechanical (pain) thresholds remained unaltered, but the hypersensitivity induced by a capsaicin injection (the pungent substance present in chili peppers) was almost completely abolished. Furthermore, these neurons were found to downregulate the K+-Cl- exporter channel KCC2, following peripheral nerve damage, resulting in increased excitability (Coull et al., 2003). It was therefore concluded that the peptidergic nociceptors-NK-1 neuronal system is important in both inflammatory and neuropathic pain conditions (Mantyh and Hunt, 2004). The non-peptidergic nociceptors terminate preferentially in the outer region of lamina IIinner, which is also called lamina IImiddle. Lamina IIinner contains relatively few neurons, one type of them being interneurons expressing Protein Kinase C γ (PKCγ). These neurons were generally believed to be innervated by the non-peptidergic nociceptors and to play an important role in neuropathic pain, since mice lacking PKCγ displayed reduced neuropathic pain behavior (Julius and Basbaum, 2001; Malmberg et al., 1997). Recent evidence however has cast doubt on this widely held assumption (Braz et al., 2005; Zylka, 2005; Zylka et al., 2005). Braz and coworkers demonstrated that non-peptidergic nociceptors bypass PKCγ neurons and terminate on other interneurons in lamina II. These neurons, in turn, project to neurons in lamina V that project directly to brain regions, such as the amygdala, the ventromedial nucleus of the hypothalamus and the bed nucleus of the stria terminalis, that may be involved in the affective-motivational component of pain. These authors

therefore concluded that there may be two systems that are responsible for the perception of pain, one is the peptidergic nociceptors-NK-1 neurons-thalamus system (spinothalamic system) that subserves the sensory-discriminative aspects of pain (e.g. where is the pain stimulus located, is the stimulus thermal or mechanical?) and the other the non-peptidergic nociceptors-lamina II-V projection neurons system that subserves the affective-motivational aspects of pain (e.g. how unpleasant is the pain?). Although this hypothesis needs to be further substantiated and although there are other pathways subserving affective-motivational aspects of pain, such as the NK-1 neurons-parabrachial area-amygdala/hypothalamus system and the NK-1 neurons-thalamus-insula/anterior cingulate cortex system, the general idea is in accordance with the "labeled lines hypothesis" of pain perception (Craig, 2002; Craig, 2003; Hunt and Mantyh, 2001), i.e. the idea that specific sensory stimuli are conveyed separately by specific anatomical and functional systems. The separate neuronal systems that process itch (McMahon and Koltzenburg, 1992), C-fiber touch (Olausson et al., 2002) and specific viscero-sensory inputs (Craig, 2003) also fit within this scheme. The labeled line hypothesis contrasts with the traditional view that the intensity and quality of the pain is coded by pattern activity of spinal cord wide dynamic range neurons, which receive both nociceptive and non-nociceptive (touch) information. This view dates back from the time of the gate control theory (Melzack and Wall, 1965)(Fig. 4), which basically proposed a gating system in the spinal cord, which closes in response to normal stimulation of fast conducting "touch" fibers, but opens when a large number of slow conducting "pain" fibers transmit sensory signals of a high intensity. The gate can be closed again if these signals are countered by renewed stimulation of the fast conducting touch fibers. According to the gate control theory it is the firing intensity and pattern of nociceptor activity and the subsequent activation of wide dynamic range neurons that determines the nature of the pain that is experienced. Although the general idea that there is an interaction between afferent inputs in the spinal cord has proven highly fruitful, the most elementary parts of the theory could not be confirmed, like e.g. presynaptic inhibition of primary afferent fibers. Increasing evidence for the existence of separate modules for different types of nociceptive information has now put the gate control theory into the background.

Following a painful stimulus associated with tissue damage, stimuli in the area of the injury and in the surrounding region that would ordinarily be perceived as slightly painful will now be perceived as significantly more so. This phenomenon is called hyperalgesia or sensitization. Two forms of sensitization exist: peripheral and central sensitization. Peripheral sensitization is caused by a decreased threshold of nociceptors for nociceptive stimuli. Peripheral sensitization generally takes place in inflammatory pain conditions and is caused by a variety of substances released by inflammatory cells and nerve terminals, the so-called "inflammatory soup". Examples include extracellular protons, adenosine triphosphate (ATP), bradykinin, histamine, serotonin, prostaglandins, tumor necrosis factor α (TNFα), interleukin 1β (IL-1β) and nerve growth factor (McMahon et al., 2005). These substances can act directly on receptors expressed by nociceptive fibers, e.g. bradykinin receptors, TrkA (Koltzenburg et al., 1999; McMahon et al., 1995), and

ion-channels, e.g. the transient receptor potential vanilloid type 1 (TRPV1) (Caterina et al., 2000; Koltzenburg, 2004) and the purinergic P2X3 receptor. These substances may also act indirectly through inflammatory cells, by enhancing the inflammatory response (Leon et al., 1994; Shu and Mendell, 1999). Receptor activation during inflammation will ultimately result in lowering of the membrane potential, thus increasing the likelihood that an action potential is triggered. An increase in the frequency of action potentials will contribute to a painful perception and at the same time enhance the release of neuropeptides from the peripheral endings of nociceptors, a process which is called neurogenic inflammation (Herbert and Holzer, 2002a; Herbert and Holzer, 2002b). A well known example of peripheral sensitization is the increased sensitivity to heat following a sunburn, which is confined to the area of the sunburn.

Central sensitization on the other hand refers to an immediate onset, activity dependent increase in the excitability of neurons in the dorsal horn of the spinal cord, following high levels of activity in the nociceptive afferents. Central sensitization is normally induced by a strong nociceptive input, e.g. after a severe injury, and is a hallmark of neuropathic pain and chronic inflammation. Like its peripheral counterpart, a number of mechanisms contribute to central sensitization. Firstly, wind-up (Dickenson, 1990; Suzuki and Dickenson, 2005) involves a progressive increase in the discharge rate of dorsal horn neurons in response to repeated low-frequency stimulation of nociceptive primary afferents. Windup arises from the summation of slow synaptic potentials that are evoked in dorsal horn neurons by nociceptive inputs. This will activate L-type calcium channels and remove the Mg^{2+} block from N-methyl-D-aspartate (NMDA) receptors, allowing Ca^{2+} ions to flow into the cell. This will increase the sensitivity of the neuron to synaptic inputs, including glutamate, the transmitter in nociceptive primary afferents. Windup, which lasts only during the period of stimulation, has been identified in volunteers that were subjected to repetitive painful electrical stimuli, and reported that the perceived intensity of the stimulus increased over time. More recently, a second form of central sensitization has been identified, which is the correlate of long-term potentiation (LTP) in the hippocampus (Ikeda et al., 2003; Willis, 2002). In contrast to wind-up, LTP may outlast the conditioning stimulus for many hours (Woolf, 1983; Woolf and Salter, 2000). Long-term changes in spinal pain transmission generally result from transcriptional changes, which may be induced by immediate early genes, e.g. c-Fos, see also Chapter 5 of this thesis. The behavioral correlate of central sensitization is mechanical allodynia, i.e. perceiving a non-noxious mechanical stimulus as painful. This phenomenon is especially pronounced in neuropathic pain conditions, when even the touch of clothing can be perceived as painful e.g. in patients with post-herpetic neuralgia.

So far only the afferent and ascending pain pathways were discussed. However the pain experience is also greatly influenced by descending controls, i.e. influences from brainstem centers that modulate spinal pain transmission (Ossipov and Porreca, 2005; Vanegas and Schaible, 2004). The concept of descending pain modulation began to take root in the 1960s, when it

was found that microinjections of morphine in the midbrain periaqueductal grey (PAG) (Tsou and Jang, 1964) and electrical stimulation of that same region (Reynolds, 1969) produced powerful antinociception. This antinociceptive effect of PAG stimulation is for a large part due to inhibition of pain transmission in the spinal cord. This inhibition is achieved by PAG projections to the rostral ventromedial medulla (RVM), which includes the Raphe Magnus nucleus, and from there through the dorsolateral funiculus to the spinal dorsal horn. It is now well established that the descending brainstem projections to the dorsal horn are not only involved in pain inhibition, but also in the facilitation of pain transmission. While opioid analgesia makes use of the inhibitory effect of descending pain modulation, pain facilitation from the RVM is thought of as a major contributor to the persistence of neuropathic pain. Recently, it was suggested that persistent ascending nociceptive inputs may contribute to descending pain facilitation, through lamina I nociceptive specific NK-1 neurons (Suzuki et al., 2002; Suzuki et al., 2004). Thus, even though the presence of a descending pain modulatory system is firmly established, the exact mechanisms that are involved in the production of pain inhibition and facilitation are still unclear.

Taken together nociceptive signals are subject to extensive modulation at all levels of the pain system, i.e. the level of the nociceptor, the level of the spinal cord, in the brainstem and in various subcortical and cortical areas, where the nociceptive signal is finally perceived. The multitude neurotransmitters, neuromodulators and receptors involved in pain transmission underlines the complexity of the system. Our knowledge of the various mechanisms is constantly increasing, but a firmly established, comprehensive view on the organization of the pain system is still lacking.

THE INVOLVEMENT OF NEUROTROPHIC FACTORS IN SENSORY TRANSMISSION

Neurotrophic factors

In the 1930's Viktor Hamburger at the University of Chicago discovered in a series of experiments that limb-bud removal and implantation in chick-embryos had a profound effect on the development of spinal cord and dorsal root ganglion (DRG) neurons (Fig. 5) (Cowan, 2001; Hamburger and Levi-Montalcini, 1949). In 1948, when it was observed that implantation of a sarcoma cell line in the body wall of chick embryos induced a striking hypertrophy of the hosts sympathetic and sensory ganglia (Bueker, 1948; Levi-Montalcini and Hamburger, 1951), it was concluded that this hypertrophic effect was probably due to a diffusible factor produced by the particular cell line. In the early 1950s the agent was named "a nerve growth-promoting factor" (Cohen et al., 1954; Levi-Montalcini and Hamburger, 1953), since at that time it was not yet clear whether it was a piece of DNA or a protein. Following isolation from snake venom

(Cohen and Levi-Montalcini, 1956) and mouse salivary gland (Cohen, 1960), the agent now termed nerve growth factor (NGF) appeared to be a protein with potent trophic and survival effects on cultured sympathetic and sensory ganglion cells (Campenot, 1981; Cohen, 1960). These findings have led Rita Levi-Montalcini, Stanley Cohen and Viktor Hamburger to postulate the "Neurotrophic Factor Hypothesis". According to the neurotrophic factor hypothesis axonal connections are first established by a larger number of neurons than is appropriate for optimal functioning of the connection. To correct for this, the target, i.e. the innervated tissue, produces a limited amount of neurotrophic factors, as a consequence of which only a limited number of neurons will survive, while other neurons will atrophy and may eventually die (Levi-Montalcini, 1987; Lewin and Barde, 1996). Although strictly speaking a neurotrophic effect only applies to an effect on neuronal survival, this term is generally interpreted to also encompass effects on neurite growth (Fig. 6) and neurotransmitter production. Neurotrophic factors are thus responsible for the correct establishment of neuronal connections in the peripheral and central nervous system and this in turn is essential for the proper function of the nervous system (Korsching, 1993; Zweifel et al., 2005). Numerous subsequent studies on the role of NGF by Levi-Montalcini and Cohen and the importance of the Neurotrophic Factor Hypothesis, which still holds true today, were awarded with the Nobel Prize in Physiology or Medicine in 1986.

Since NGF affects only a specific population of peripheral neurons, i.e. those neurons expressing TrkA, the receptor for NGF (see later), it was presumed that other neurotrophic factors must exist that would support growth and survival of other populations of neurons. However it was not until the 1980's that another in vivo neurotrophic factor, Brain-Derived Neurotrophic Factor (BDNF) was discovered, by purification from pig brain (Barde et al., 1982; Hofer and Barde, 1988; Leibrock et al., 1989). Based on their structural homology, three other neurotrophic factors, i.e. neurotrophin-3 (NT-3) (Hohn et al., 1990), NT4/5 (Hallbook et al., 1991) and NT-6 (Gotz et al., 1994) were then cloned, which together with NGF and BDNF make up the neurotrophin family.

Apart from the neurotrophins, various other proteins with trophic effects on neurons exist. Ciliary neurotrophic factor (CNTF), originally found to affect ciliary ganglion parasympathetic neurons (Barbin et al., 1984; Lin et al., 1989), also affects dorsal root ganglion neurons and central nervous system neurons (Hagg et al., 1992). Furthermore, although their effects are not restricted to neurons, Leukemia Inhibitory Factor (LIF) (Martinou et al., 1992; Murphy et al., 1991) and Fibroblast Growth Factors (Eckenstein et al., 1990; Hatten et al., 1988) also affect neurons in both the peripheral and central nervous system.

In 1993, Glial cell line-Derived Neurotrophic Factor (GDNF) was discovered (Lin et al., 1993), as the first member of what later turned out to be a whole new group of neurotrophic factors called the GDNF family ligands (GFLs). GFLs are distant members of the Transforming Growth Factor-β super family. GDNF was isolated and cloned by virtue of its strong effect on survival, differentiation and dopamine uptake of embryonic midbrain dopaminergic neurons. The GFLs

also comprise Neurturin (NRTN) (Kotzbauer et al., 1996), Persephin (PSPN) (Milbrandt et al., 1998) and Artemin (ARTN) (Baloh et al., 1998). Apart from effects on nerve cells, most notably dopaminergic cells, motoneurons, dorsal root ganglion (DRG) neurons and sympathetic neurons, the GFLs also have important functions outside the nervous system (Airaksinen and Saarma, 2002; Meng et al., 2000; Schuchardt et al., 1994). As a result of its strong survival effects on cultured midbrain dopaminergic (Lin et al., 1993) cells and motoneurons (Henderson et al., 1994), GDNF has been extensively tried and found successful in animal models of Parkinson's disease (Behrstock et al., 2005) and amyotrophic lateral sclerosis (Bohn, 2004). However, phase I clinical trials with intrathecal GDNF in patients with Parkinson's disease and ALS were discontinued because of a lack of efficacy.

Receptors for neurotrophic factors

Neurotrophins signal through the tropomyosin receptor kinase (Trk) family of protein kinases (Barbacid, 1995). Whereas NGF binds to TrkA, BDNF and NT-4/5 interact with TrkB. NT-3 appears to be more promiscuous and binds to each of the Trk receptors, but its primary biological responses are mediated through TrkC (Fig. 7). In addition, neurotrophins interact with a second receptor, p75, a member of the tumor necrosis factor super family (Chao, 1994). Neurotrophins, which bind to Trk receptors with high-affinity, induce dimerization of Trk receptors and these activated receptor complexes consequently induce cell survival, neurite outgrowth and neuronal differentiation. In contrast to Trks, neurotrophins bind to p75 with much lower affinity, which has led to speculation that neurotrophins represent only partial ligands of p75 (Ibanez, 2002). More recently, pro-neurotrophins, the precursor molecules of the neurotrophins, have been discovered as the high affinity ligands for the p75 receptor (Ibanez, 2002; Lee et al., 2001; Teng et al., 2005). Activation of p75 by pro-NGF (Lee et al., 2001) and pro-BDNF (Teng et al., 2005) results in apoptotic cell death (Frade et al., 1996), i.e. the opposite effect of Trk activation. *In vivo* however, since the affinity of Trk for neurotrophins is many times greater than that of p75, the trophic effects of the neurotrophins usually predominate (Chao et al., 1998).

The GFLs, on the other hand, signal through a completely different mechanism. In 1996 the proto-oncogene Rearranged during Transfection (RET) was discovered as the signaling receptor for GDNF (Durbec et al., 1996; Trupp et al., 1996). Later on it appeared that this tyrosine kinase receptor was the common signaling receptor for all GFLs. RET is activated only if the GFL is first bound to a GDNF-family receptor-α (GFRα). Four different GFRα receptors have been characterized (GFRα1-4), which determine the ligand specificity of the GFRα-RET complex. GDNF binds to GFRα1, then forms a complex with RET (Jing et al., 1996; Treanor et al., 1996). NRTN binds to GFRα2, ARTN to GFRα3 and PSPN activates RET by binding to GFRα4. NRTN and ARTN might crosstalk weakly with GFRα1, and GDNF with GFRα2 and GFRα3. In mammals at least PSPN can only bind to GFRα4 (Airaksinen and Saarma, 2002; Airaksinen et al., 1999) (Fig.

8). Although most *in vivo* biological effects of the GFLs can be attributed to GFL-RET signaling, *in vitro* experiments have shown RET-independent signaling through GFRα1 (Poteryaev et al., 1999; Trupp et al., 1999) and effects through interactions of GDNF with neuron cell adhesion molecule (NCAM) (Paratcha et al., 2003; Sariola and Saarma, 2003). Additionally, it has recently been shown that RET can be activated by NGF, without interference of any of the GFRαs (Tsui-Pierchala et al., 2002)

Trophic effects of neurotrophic factors on sensory neurons

During development of the nervous system, once proper synaptic connections are established, according to the Neurotrophic Factor Hypothesis, neurons remain dependent on the presence of their targets for continued survival and differentiation. In the adult nervous system, target-derived neurotrophic factors provide support to damaged neurons, addition of neurotrophic factors to damaged neurons may enhance their regeneration (Goldberg and Barres, 2000). However the Neurotrophic Factor Hypothesis needs to be updated in this respect that peripheral tissues (i.e. the targets) are not the only source of neurotrophic factors, since they have also been found to be released from supportive cells, other neurons and even the dependent neurons themselves, the latter via so-called autocrine signaling (Korsching, 1993). The above-described paradigm of neurotrophic factor functioning during development and in the adult holds true explicitly for sensory neurons.

NGF supports a subclass of small diameter nociceptive primary afferent sensory neurons during prenatal development (Fitzgerald, 2005). Depletion of NGF in knock-out animals results in a 70% reduction of DRG neurons (Crowley et al., 1994) and an increase in the number of these neurons occurs in NGF over-expressing mice (Albers et al., 1994; Jhaveri et al., 1996). DRG neurons are also dependent on other neurotrophins, like NT-3 (Albers et al., 1996; Ernfors et al., 1994; Farinas et al., 1994) and BDNF (Jones et al., 1994). Strong evidence exists that some of these neurons require more than one neurotrophin to survive into adulthood (Mendell et al., 1999). In most cases, neurotrophins appear to modulate cell numbers by regulating programmed cell death in the prenatal period (Farinas et al., 1996). Apart from an effect as a prenatal survival factor, NGF is also essential for normal postnatal development of the phenotype of nociceptors, as has been shown by reduced numbers of high-threshold mechanoreceptor afferents in animals that were treated postnatally with an antibody to NGF (Lewin et al., 1992; Mendell et al., 1999; Ritter et al., 1991), while total numbers of nociceptors remained unaltered. In early postnatal life about half of the neurons that are NGF dependent undergo a switch, changing from NGF to GDNF dependence (Molliver et al., 1997). The effects of absence of GDNF on nociceptor survival and development have not been studied, because animals lacking GDNF or RET have renal agenesis and die soon after birth (Schuchardt et al., 1994).

In adulthood neurotrophic factors may contribute to regeneration following nerve damage. One striking peculiarity in higher vertebrates is the ability of peripheral nervous system (PNS) but not of central nervous system (CNS) neurons to regenerate their axons, which has already been noted by Ramón y Cajal in 1928 (Ramon y Cajal, 1928). Although much research has focused on inhibitory signals by CNS glia and more recently on the presence of growth-inhibitory molecules associated with myelin (Domeniconi and Filbin, 2005), loss of trophic stimuli needed to promote the survival and regeneration of axotomized neurons may also play an important role. As far as the sensory system is concerned, NGF, NT-3 and GDNF (but not BDNF) have all been shown (Ramer et al., 2002; Ramer et al., 2000) to promote growth of severed dorsal root axons across the Dorsal Root Entry Zone (DREZ), an area of the CNS which normally acts as a barrier to regenerating axons. In the peripheral nervous system NGF (Verge et al., 1995), BDNF (Acheson et al., 1995), GDNF (Bennett et al., 1998) and ARTN (Gardell et al., 2003) have all been shown to prevent axotomy-induced degenerative changes in DRG neurons bearing their respective receptors, and in the case of GDNF and ARTN to cause a reduction in neuropathic pain behavior in experimental animals. Although NGF has been tried in patients with diabetic neuropathy (Apfel, 2002; Apfel et al., 2000), no beneficial effects from NGF on the clinical condition of those patients has been described.

Involvement of neurotrophic factors in modulation of nociceptive information: NGF as an inducer of inflammatory hyperalgesia

In contrast to the survival effects of neurotrophic factors on DRG neurons during development in utero and the effects on normal phenotypic development of DRG neurons in the early postnatal period, the physiological role of neurotrophic factors changes dramatically during adulthood. In 1993 for the first time (Lewin et al., 1993), more than 40 years after the discovery of NGF as a trophic factor for sensory neurons, an acute effect of NGF as a contributor to inflammatory hyperalgesia has been described (Lewin and Barde, 1996; Lewin and Mendell, 1993) and subsequent papers (Koltzenburg et al., 1999; Lewin et al., 1994; McMahon et al., 1995; Woolf et al., 1994) have confirmed this. Inflammation induces both thermal and mechanical hyperalgesia. Thermal hyperalgesia is a form of primary hyperalgesia and arises from increased firing of high threshold C- and Aδ-nociceptor neurons. Primary hyperalgesia is usually confined to the area of tissue damage or inflammation, but may extend slightly beyond its borders, and is mediated by peripheral mechanisms, such as neurogenic inflammation caused by antidromic release of inflammatory mediators such as CGRP and SubP (Herbert and Holzer, 2002a; Herbert and Holzer, 2002b). The area of primary hyperalgesia is usually demarcated by a red flare. Pain evoked by stimulating tissue that is undamaged or unaffected by disease is termed secondary hyperalgesia and manifests itself as mechanical hyperalgesia. The extend of the area of secondary hyperalgesia is not determined by cutaneous processes such as the production of flare and involves

the activation of Aβ fibers that normally convey innocuous sensory information to the spinal cord. Following induction of inflammation in the skin of adult animals, endogenous NGF released by mast cells (Woolf et al., 1996) and other inflammatory cells, like activated macrophages and polymorphonuclear leucocytes (Sofroniew et al., 2001), induces both heat hyperalgesia and mechanical hyperalgesia (Lewin et al., 1994). It has been shown that NGF is both necessary (Koltzenburg et al., 1999) and sufficient (Lewin et al., 1993) for the induction of inflammatory hyperalgesia. NGF induced heat hyperalgesia is mediated by peripheral mechanisms, such as up regulation of the neuropeptides SubP (Woolf et al., 1994) and CGRP (McMahon et al., 1995) in DRG neurons and activation of p38 mitogen-activated protein kinase (MAPK), resulting in an upregulation of the capsaicin receptor transient receptor potential vanilloid type-1 (TRPV-1) (Ji et al., 2002). NGF induced mechanical hyperalgesia is mediated by central mechanisms, such as the upregulation of BDNF in DRG neurons (Apfel et al., 1996; Shu and Mendell, 1999) and the sensitization of NMDA receptors (Lewin et al., 1994).

Analogous to the role of endogenous NGF in modulation of inflammatory pain, a role for NGF as an inducer of hyperalgesia in cancer pain has more recently been described (Halvorson et al., 2005; Jongen et al., 2002; Sevcik et al., 2005). Not surprisingly, one of the tumors used to induce bone cancer was a sarcoma cell-line (compare (Bueker, 1948), which secretes high levels of NGF (Jongen et al., 2002; Sevcik et al., 2005). In bone cancer, a NGF sequestering antibody was shown to reduce spontaneous pain behavior as well as heat (Halvorson et al., 2005) and mechanical hyperalgesia (Sevcik et al., 2005). The effect of anti-NGF on spontaneous cancer pain behavior was even stronger than high-dose systemic morphine (Halvorson et al., 2005). The mechanisms by which NGF may contribute to bone cancer pain have not been fully elucidated. A similar mode of action as in inflammatory pain may apply, since tumors generally contain a substantial number of tumor associated inflammatory cells (Jongen et al., 2002). In addition, NGF may modulate cancer specific pain mechanisms, since osteoclasts and many cancer cell types express the receptor components for NGF signaling (Descamps et al., 2001; Miknyoczki et al., 2002; Missale et al., 1998) and effects of NGF and antagonists on tumor growth have been described in many cancers *in vitro* (Benini et al., 1999; Missale et al., 1998; Oelmann et al., 1995) and *in vivo* (George et al., 1999; Miknyoczki et al., 2002). As a result of the latter, it is remarkable that no differences in tumor growth or bone destruction were detected in bone cancer in anti-NGF treated animals as compared to controls (Halvorson et al., 2005; Sevcik et al., 2005).

In contrast to extensive data on the role of NGF in inflammatory and more recently cancer pain, relatively weak evidence for a role of NGF as a modulator in neuropathic pain exists. NGF has been shown to modulate neuropeptide (Verge et al., 1995) and BDNF (Fukuoka et al., 2001) expression in DRG neurons following nerve injury and an anti-NGF blocking antibody attenuated heat hyperalgesia (Ro et al., 1999) and mechanical hyperalgesia (Ren et al., 1995) following chronic constriction injury of the sciatic nerve.

Involvement of neurotrophic factors in modulation of nociceptive information: BDNF as a modulator of centrally mediated hyperalgesia

While NGF is not produced in neurons, but derived from peripheral sources such as skin, Schwann cells and DRG satellite cells (Sofroniew et al., 2001), BDNF is produced in a subset of DRG neurons that also express TrkA and neuropeptides like SubP and CGRP (Michael et al., 1997; Wetmore and Olson, 1995; Zhou et al., 1999). Following inflammation (Apfel et al., 1996; Mannion et al., 1999) and nerve injury (Fukuoka et al., 2001), BDNF is upregulated in DRG neurons in a NGF-dependent fashion. This up regulation is mediated through TrkA, which is present in the same DRG neurons that also express BDNF. TrkA and BDNF expressing DRG neurons belong to the peptidergic primary afferents. Within this subgroup of DRG neurons, BDNF is anterogradely transported towards terminals in the superficial spinal dorsal horn (Holstege et al., 1997; Tonra et al., 1998; Zhou and Rush, 1996), where it is contained in dense-cored vesicles (Michael et al., 1997). Dense-cored vesicles contain catecholamines and peptide neurotransmitters, like CGRP and SubP, in contrast to synaptic vesicles, which contain small molecule neurotransmitters such as gamma-amino butyric acid (GABA), glutamate and acetylcholine. Substances in dense-cored vesicles behave differently from those in synaptic vesicles, in the sense that they are only released following strong and prolonged stimulation. The presence of BDNF mRNA in DRG neurons, the anterograde transport within the dorsal root, the localization of BDNF in dense-cored vesicles in terminals and the presence of TrkB (Yan et al., 1997) on its postsynaptic target, i.e. local and projection neurons in the superficial dorsal horn, all point to a role for BDNF as a neurotransmitter in spinal pain transmission (Pezet et al., 2002b). Such a role has actually been demonstrated for BDNF in the spinal cord, where BDNF increases nociceptive spinal reflex excitability (Kerr et al., 1999) and is responsible for the mechanical hyperalgesia following injection of several inflammation inducing substances (Kerr et al., 1999; Mannion et al., 1999; Thompson et al., 1999). Although the exact mechanism of action of BDNF in spinal cord signaling is not known, it has been shown that BDNF induces activation of the signaling kinases Extracellular Signal-regulated Kinase (ERK) and Phospho Kinase C (PKC) via TrkB (Lever et al., 2003; Pezet et al., 2002a; Slack et al., 2004), resulting in subsequent phosphorylation of the *N*-methyl-d-aspartate (NMDA) receptor subunit 1 (NR1) (Slack et al., 2004) and transcription of the immediate early gene c-fos (Kerr et al., 1999). The effects of BDNF on ERK phosphorylation have been demonstrated more specifically in TrkB expressing spinothalamic tract projection neurons (Slack et al., 2005). Aside from the spinal cord, a role for BDNF as a neurotransmitter has also been described in other systems, like the hippocampus, where BDNF is involved in long-term potentiation (Figurov et al., 1996; Kang et al., 1997; Korte et al., 1995; Poo, 2001) and learning (Minichiello et al., 1999). Interestingly, proBDNF, acting through the p75 receptor, has exactly the opposite effect of BDNF, i.e. enhancement of long term depression (Woo et al., 2005), just like the opposite effects that have been described earlier for NGF and proNGF. At least in the brain it was shown that the $Na_v1.9$ sodium channel underlies the fast BDNF-evoked excitation through TrkB (Blum et al., 2002; Rose et al., 2004).

In addition to an effect from primary afferent BDNF on spinal pain neurons, recent evidence suggests that BDNF released from neurons originating in the periaqueductal gray acts on descending rostral ventromedial medulla neurons, thereby inducing facilitation of nociceptive transmission (Guo et al., 2006). In contrast to its role in modulating nociceptive, i.e. inflammatory, transmission, BDNF from DRG neurons does not seem to be involved in the modulation of neuropathic pain (Zhao et al., 2006). However, BDNF released from spinal microglia (Nakajima et al., 2002) has been shown to induce allodynia in a neuropathic pain model (Coull et al., 2005), in line with a growing notion that spinal microglia plays an important role in the generation and maintenance of neuropathic pain (Tsuda et al., 2005).

GDNF AS A POTENTIAL MODULATOR OF SPINAL PAIN TRANSMISSION-AIM OF THE THESIS

Although GDNF has traditionally been considered a trophic factor for a specific subpopulation of nociceptors (Bennett et al., 1998; Molliver et al., 1997), our finding that GDNF protein is strongly expressed in primary afferent terminals in the dorsal horn and the anterograde transport within dorsal root fibers was the first indication that GDNF might have other roles than a trophic role in the spinal dorsal horn (Holstege et al., 1998). Since at that time a role for BDNF as a neuromodulator of spinal nociceptive transmission had just been proposed, we hypothesized that GDNF may act in a similar way, based on the identical anatomical localizations of BDNF and GDNF in the spinal cord and their presence within dense-cored vesicles (Holstege et al., 1999; Michael et al., 1997; Ohta et al., 2001). We therefore set out a series of anatomical and functional experiments, which are described in this thesis, to provide additional evidence for such a role of GDNF. Following the anatomical localization of GDNF protein in the spinal dorsal horn (Chapter 2), we subsequently studied its regulation following peripheral nerve transection (Chapter 3). We then studied the effects of intrathecally injected GDNF on immediate early gene expression in spinal dorsal horn neurons and compared the magnitude of the effect with that of BDNF and NMDA (Chapter 4). Immediate early gene expression in dorsal horn neurons was used as a marker for activation of these neurons by the intrathecally injected substances. Finally, the anatomical and functional expression of RET, the main signaling receptor for GDNF, was studied, both in the normal situation and following nerve injury (Chapter 5).

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FIGURES

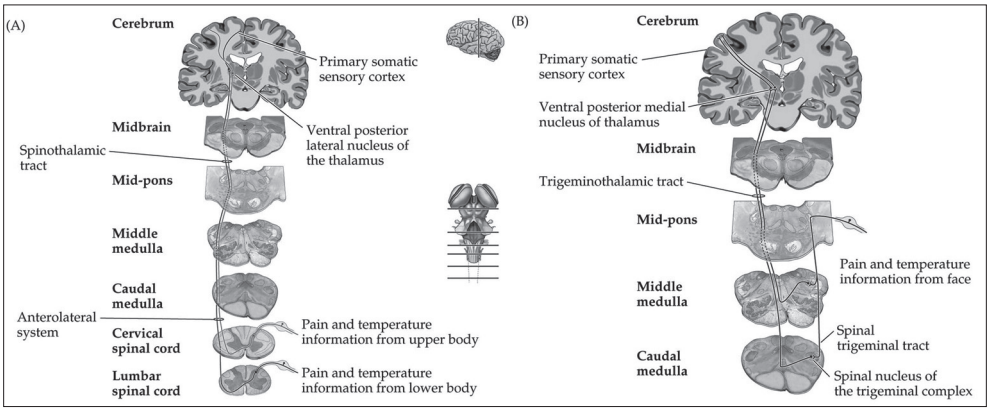


Fig. 1. The sensory component of pain. Major peripheral and central pathways for the discriminative aspects of pain and temperature sensation, which are perceived by the same systems. (A) The spinothalamic or anterolateral system, which carries information about these sensations from the body and extremities. (B) The trigeminal pain and temperature system, which carries information about these sensations from the face.

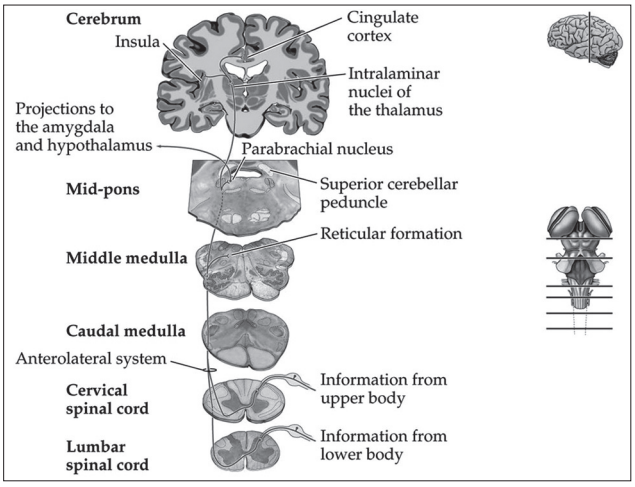


Fig. 2. The affective-motivational component of pain. Nociceptive information critical for signaling the unpleasant quality of pain is mediated by projections to the reticular formation (including the parabrachial nucleus) and to the intralaminar nuclei of the thalamus. From the latter tertiary neurons project to the cingulate cortex and insula. Parabrachial neurons on the other hand project to the amygdala and hypothalamus, but also to the periaqueductal gray, a structure that plays an important role in the descending control of activity in the pain pathway.

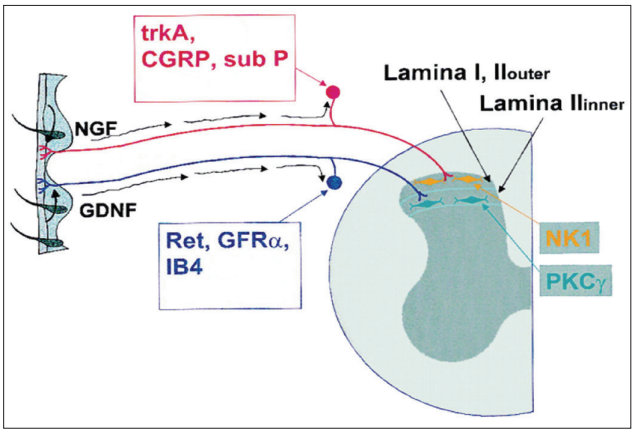
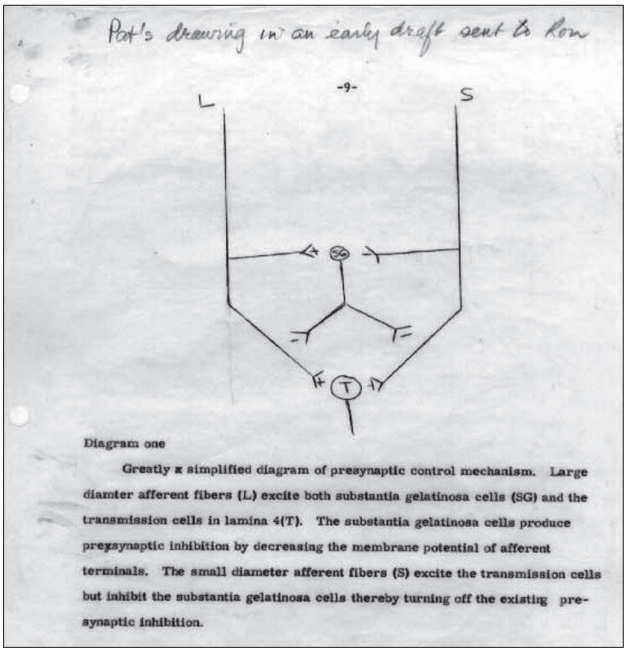


Fig. 3. Diagram showing the two subclasses of C nociceptors, peptidergic nociceptors in red and non-peptidergic nociceptors in blue. The peptidergic nociceptors are dependent on NGF during development, express the receptor for NGF, TrkA, and neuropeptides, like CGRP and SubP, and terminate in lamina I and II_{outer}, the most superficial layers of the dorsal horn. An important central target of the peptidergic C nociceptors are nociceptive specific spinal projection neurons that express Neurokinin-1 (NK-1), the receptor for SubP. The non-peptidergic nociceptors are dependent on GDNF during development, express the receptor components for GDNF, RET and GFRα-1, and the plant lectin Isolectin B4 (IB4) and terminate in lamina II_{inner}. An important central target of the non-peptidergic C nociceptors are interneurons that express the γ isoform of protein kinase C (PKCγ).


Pat's drawing in an early draft sent to Ron

-9-

L S

T

Diagram one

Greatly a simplified diagram of presynaptic control mechanism. Large diamter afferent fibers (L) excite both substantia gelatinosa cells (SG) and the transmission cells in lamina 4(T). The substantia gelatinosa cells produce presynaptic inhibition by decreasing the membrane potential of afferent terminals. The small diameter afferent fibers (S) excite the transmission cells but inhibit the substantia gelatinosa cells thereby turning off the existing pre-synaptic inhibition.

Fig. 4. The gate control theory

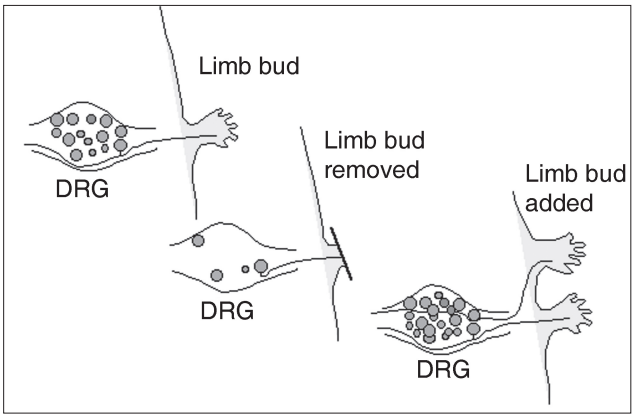


Fig. 5. The history of NGF goes back to work by Samuel Detwiler, Viktor Hamburger and others in the 1920's and 1930's. They showed firstly that neuronal cell death occurred in the dorsal root ganglia of normally developing embryos, and that the number of sensory neurons which survived into adulthood depended on the size of the target which they innervated. In amphibians, the number of sensory neurons in the DRG could be reduced by removing the normal target, while it could be increased by transplanting an additional limb bud. This was found to be due to changes in the survival of postmitotic neurons and not in the division of neuronal precursor cells.

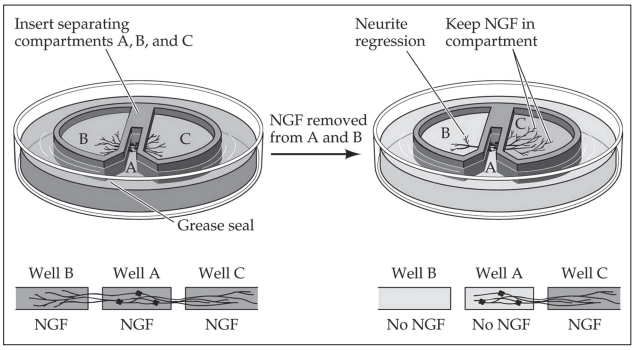


Fig. 6. Experiment by (Campenot, 1981) showing that NGF can influence neurite outgrowth by local action. Three compartments of a culture disc (A, B, C) are separated from one another by a plastic divider sealed to the bottom of the disc by grease. Isolated rat sympathetic ganglion cells plated in compartment A can grow through the grease seal and into compartments B and C. (A magnified view looking down on the compartments is shown below.) Growth into a lateral chamber occurs as long as the compartment contains an adequate concentration of NGF. Subsequent removal of NGF from a compartment causes a local regression of neurites in the other compartments.

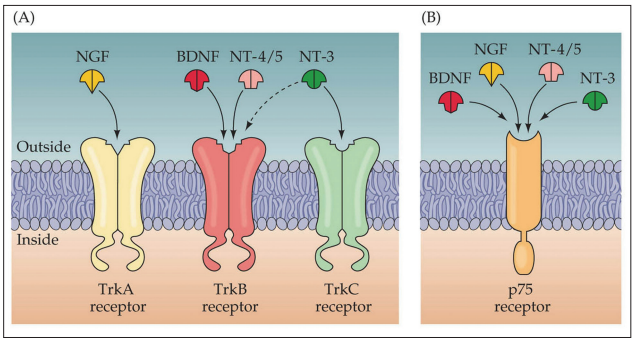

(A)
(B)
NGF
BDNF
NT-4/5
NT-3
Outside
Inside
TrkA receptor
TrkB receptor
TrkC receptor
p75 receptor

Fig. 7.

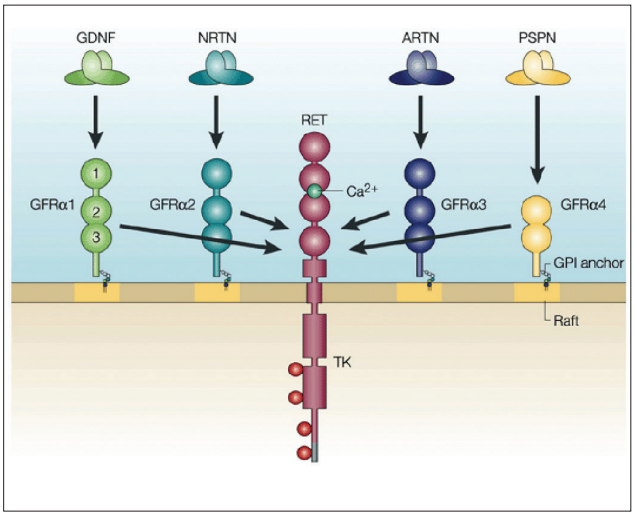

GDNF
NRTN
ARTN
PSPN
RET
GFRα1
GFRα2
GFRα3
GFRα4
Ca2+
GPI anchor
Raft
TK

Fig. 8.

Fig. 7. (previous page) Neurotrophin receptors and their specificity for the neurotrophins. (A) The Trk family of receptor tyrosine kinases for the neurotrophins. TrkA is primarily a receptor for NGF, TrkB a receptor for BDNF and NT-4/5, and TrkC a receptor for NT-3. Because of the high degree of structural homology among both the neurotrophins and the Trk receptors, there is some degree of cross-activation between factors and receptors. For example, NT-3 can bind to and activate TrkB, as indicated by the dashed arrow. These distinct receptors allow various neurons to respond selectively to the different neurotrophins. (B) The p75 low-affinity neurotrophin receptor binds all neurotrophins at low affinities (as its name implies). This receptor confers the ability to respond to a broad range of neurotrophins upon fairly broadly distributed classes of neurons in the peripheral and central nervous system.

Fig. 8. (previous page) GDNF-family ligands and receptor interactions. Homodimeric GDNF-family ligands (GFLs), i.e. GDNF, NRTN, ARTN, PSPN, activate RET tyrosine kinase by first binding their cognate GDNF-family receptor-α (GFRα) receptors. Arrows indicate the preferred ligand-receptor interactions that are known to occur physiologically *in vivo*. GFRα proteins are attached to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor. GFLs bind mainly to the second domain of GFRα receptors, which is also crucial for RET binding. Binding of Ca^{2+} ions to one of the four extracellular cadherin-like domains of RET is required for its activation by GFL-GFRα complexes. Phosphorylation of tyrosine residues (red balls) in the RET intracellular part induces several intracellular signaling cascades.

Chapter 2

Immunocytochemical localization of GDNF in primary afferents of the lumbar dorsal horn

Running Title:

GDNF is localized in primary afferents

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ABSTRACT

Immunocytochemistry was used to identify glial cell line-derived neurotrophic factor (GDNF) in rat spinal cord. Strong GDNF labeling was found in fibers and terminals in laminae I and II (outer) and to a lesser extent in the remaining laminae. A few spinal ganglion cells also contained GDNF. After dorsal root transection GDNF disappeared from the dorsal horn and after dorsal root ligation there was accumulation of GDNF only on the ganglion side of the ligation. These findings demonstrate anterograde transport of GDNF within primary afferent fibers, which constitute the only source of GDNF labeling in the dorsal horn. The strong presence of GDNF in the superficial dorsal horn may indicate that GDNF has a role in pain transmission in the adult rat spinal cord.

Keywords: Dorsal horn, Glial cell line-derived neurotrophic factor, Immunocytochemistry, Pain, Rat, Rhizotomy, Spinal cord, Spinal ganglion

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) was originally purified and cloned as a survival factor for midbrain dopaminergic neurons.[1] GDNF was shown to be able to protect adult dopaminergic neurons against various toxic substances or axotomy.[2-6] In addition, similar to many other neurotrophic factors, GDNF rescues spinal motoneurons from dying after axotomy of a peripheral nerve in the neonatal period[7,8] and may also play a role in the development and maintenance of sensory neurons.[9,10] Injections of [*125*I]-GDNF showed retrograde transport of GDNF from the striatum to the nigra and from the periphery to the spinal ganglion,[9,11] suggesting that in these systems GDNF acts as a retrogradely transported neurotrophic factor. Two other neurotrophic factors, neurturin and persephin, which are about 40% identical to GDNF, have recently been identified.[12] Together with GDNF they constitute a separate family of neurotrophic factors, distantly related to the transforming growth factor-β superfamily, but also showing characteristics of the neurotrophin family.[13]

A tyrosine kinase receptor, known as Ret, was identified recently as the receptor for GDNF. However, for signal transduction at the Ret receptor an additional extracellular GDNF-binding protein, named GFRα, is necessary, several of which have now been identified.14 Areas expressing mRNA encoding for GDNF and its various receptor components have been identified in the adult central nervous system. GDNF mRNA was found in specific areas of neocortex, thalamus, hippocampus, cerebellum, mesencephalon, pons medulla and spinal cord,15-17 while levels in dorsal root ganglia were low.18,19 The Ret and GFRα receptor mRNAs were also found in the majority of these areas, including dorsal root ganglia and the spinal dorsal horn.10,20,21

So far, the localization of the GDNF protein has not been studied in the central nervous system. We have used immunocytochemistry to localize GDNF protein in the rat spinal cord. Since GDNF immunoreactive fibers and terminals are abundantly present in the superficial dorsal horn, we have also used dorsal root transection or ligation for identifying the origin and the direction of transport of GDNF.

MATERIALS AND METHODS

For GDNF immunocytochemistry 12 male Wistar rats were deeply anesthetized with pentobarbital and perfused transcardially with 250 ml phosphate buffer (PB; 0.025M, pH 7.3), containing 0.8% NaCl, 0.8% sucrose and 0.4% D-glucose followed by 400 ml of PB (0.05M) containing 4% paraformaldehyde and 400 ml of the same fixative also containing 15% sucrose, both at 4°C. After perfusion, the spinal cord and the L4 and L5 spinal ganglia were removed and postfixed at 4°C for 2 hours in fixative containing 15% sucrose. Frozen sections were cut at 40 μm, rinsed in Tris-buffered saline (TBS) and processed for immunocytochemistry (all steps at room temperature

except where indicated otherwise). The sections were preincubated in 10% normal goat serum (NGS) in TBS containing 0.4% Triton X-100 and then transferred to TBS containing 1% NGS, 0.1% Triton X-100 and a GDNF antibody diluted 1:1500-3000 and incubated approximately 40 hours at 4°C. Sections were then rinsed (4x) in TBS, incubated for 90 min in biotinylated goat-anti-rabbit (1:200 in TBS also containing 0.1% Triton X-100 and 1% NGS), rinsed again (TBS; 4x), processed with the ABC method (Vector Elite) and reacted with 0.025% diaminobenzidine (DAB), containing 0.005% hydrogen peroxide. The GDNF antibody (Santa Cruz sc-328) is a polyclonal antibody directed against amino-acids 186-205 of the precursor form of human GDNF. For controls, some sections were processed as above, except that the primary GDNF antibody was either omitted and substituted with TBS, or preincubated (2 h) with a 10-fold (by weight) excess of peptide antigen (Santa Cruz sc-328 P) in TBS.

Five rats were deeply anesthetized with 2% halothane in a mixture of O_2 and N_2O. A laminectomy was performed at L3, after which the L3-S1 dorsal roots, which pass at this level, were transected. After closing the wound the animals survived for 10 days. They were then deeply anesthetized with pentobarbital and perfused as above. The spinal cord was removed and processed for GDNF immunocytochemistry as above. For dorsal root ligation a similar procedure was used for exposing the L3-S1 dorsal roots. One or two of these roots were tightly ligated using a 6.0 nylon thread. Two of these animals survived for 1 day and 4 animals survived for 9 days. After perfusion, the dorsal roots were removed and embedded in gelatin, after which frozen sections were cut and treated for GDNF immunocytochemistry as above.

RESULTS

Light microscopical examination of the lumbo-sacral spinal cord sections processed for GDNF immunocytochemistry showed very dense reaction product in fibers and presumed terminals of the superficial dorsal horn (laminae I and outer lamina II). A few labeled varicose fibers were also seen in laminae II (inner), III, IV and in the central and lateral parts of lamina V. Occasionally these fibers appeared to form a sort of cluster, centrally in laminae IV and V (Fig. 1). In the medial part of the dorsal horn, at the border between the gray and white matter, a narrow band of strongly labeled fibers was seen, which seemed to terminate in the area dorsal to the central canal. This was especially clear at low lumbar and sacral levels, where the area dorsal to the central canal becomes larger. Labeled fibers were also present in the lateral spinal nucleus and in the sacral parasympathetic nucleus. The remaining laminae showed only a few lightly labeled fibers. Labeled neurons were not observed.

In the spinal ganglia several ganglion cells showed a variable amount of faint granular labeling, but only very few cells were unambiguously labeled and quantification was therefore not

attempted. Labeled ganglion cells were small or medium sized and occasionally were observed to give rise to an immunoreactive fiber that could be followed over some distance (Fig. 2). Immunoreactive fibers, often showing fine immunoreactive granules, were seen throughout the ganglion.

In order to determine whether GDNF immunoreactive fibers in the dorsal horn were dorsal root fibers, a rhizotomy was performed by cutting the lumbo-sacral dorsal roots, leading to primary afferent degeneration in the spinal cord. In these cases, the strongly GDNF-immunoreactive fibers in the superficial dorsal horn and in laminae III-V as well as those dorsal to the central canal nearly all disappeared in the corresponding segments on the rhizotomized side. A few weakly labeled fibers usually remained, mainly in the superficial dorsal horn. In contrast, the same area on the non-rhizotomized contralateral (control) side was still strongly labeled (Fig. 3). The labeled fibers in the sacral parasympathetic nucleus also disappeared after a lumbosacral rhizotomy, while the limited number of lightly stained fibers in the ventral horn remained on the rhizotomized side as on the contralateral side.

The dorsal roots that were processed for GDNF immunocytochemistry showed several-GDNF immunoreactive fibers at the ganglion side of the ligation (Fig. 4), both at 1 and 9 days survival after the ligation. The spinal side of a ligation never showed a significant amount of GDNF immunoreactivity.

Control sections, incubated with TBS or with GDNF antibody pre-incubated with peptide antigen did not show immunoreactivity, strongly suggesting that the antibody only recognized the GDNF-specific peptide sequence.

DISCUSSION

In this study we have used immunocytochemistry with a highly specific antibody against GDNF to identify the distribution of GDNF protein in the lumbo-sacral spinal cord and corresponding ganglia. The GDNF-immunoreactive fibers and presumed terminals were especially strong in the superficial dorsal horn, with some varicose fibers in the deeper laminae and the area around the central canal. This characteristic pattern of labeling is also observed with various neuropeptides like calcitonin gene-related peptide (CGRP), somatostatin, substance P and galanin.[22] However, some of these neuropeptides are also present in neurons of the dorsal horn, while we never observed neuronal labeling with GDNF in the spinal cord. Studies using *in situ* hybridization or the more sensitive reverse transcription-polymerase chain reaction have shown that neurons expressing GDNF mRNA in the adult spinal cord[15-17, 19] are motoneurons and interneurons of the ventral horn, rather than the dorsal horn.[16, 23] The lack of neuronal labeling that we observed in the spinal cord, may indicate that the GDNF protein level in neuronal somata is too low for

detection with immunocytochemistry, either because only low amounts of GDNF are produced or because GDNF is transported away from the soma immediately after production. The latter situation may also apply for the spinal ganglion cells, which were only weakly immunoreactive, while their (presumed) terminals in the dorsal horn were strongly immunoreactive, probably due to the accumulation of GDNF in these terminals. A similar phenomenon occurs with the localization of various peptides, in which cases colchicine an be used to increase neuronal labeling.[22] We are currently exploring the possibility to enhance neuronal labeling for GDNF after colchicine treatment.

In order to investigate the origin of the GDNF fibers, dorsal rhizotomies were performed, which resulted in a dramatic decrease of GDNF immunolabeling in the dorsal horn. This strongly suggests that GDNF in the dorsal horn is present exclusively in primary afferent fibers and their terminals and is not derived from spinal or supraspinal sources. The few fibers that remained in the dorsal horn after a rhizotomy were probably derived from distant intact dorsal roots, which are known to travel long distances in the spinal cord. However, another origin of these fibers, e.g. from local interneurons, cannot be excluded on the basis of our data. The experiments using ligation of a dorsal root showed that GDNF accumulated at the ganglion side of a ligation. This finding suggests that GDNF is anterogradely transported in primary afferent fibers towards their terminals in the dorsal horn. It seems most likely that the transported GDNF is produced in the ganglion cells, although in our material only few weakly labeled ganglion cells were observed. Immunocytochemical studies on human post mortem tissue, however, showed small to medium sized ganglion cells that were strongly immunoreactive for GDNF.[24] This would be in agreement with in situ hybridization data showing that spinal ganglion cells produce GDNF mRNA in relatively low amounts in the adult,[19] in contrast to the early neonatal period when GDNF mRNA levels were higher.[15] Taken together, our data indicate that GDNF, produced in dorsal root ganglia, is transported anterogradely to the terminals of primary afferent fibers in the dorsal horn. However, it cannot be excluded that GDNF, produced in the periphery, is transported transganglionically to primary afferent terminals in the dorsal horn.

Our findings on the spinal distribution and transport of GDNF very much resemble those of brain derived neurotrophic factor (BDNF), a member of the neurotrophin family. Both substances show the strongest labeling in the superficial dorsal horn, disappear after dorsal rhizotomy and accumulate at the ganglion side of a ligation.[25, 26] Furthermore, BDNF was found within dense cored vesicles,[26, 27] suggesting activity dependent release of BDNF. The localization in dense cored vesicles explains the accumulation after a ligation and the relative weak labeling of ganglion cells, since dense cored vesicles are produced in the cell soma and subsequently transported to the terminal. The granular appearance of the labeled fibers observed in the ganglion may also indicate localization in dense cored vesicles. Obviously, electron microscopy is needed to resolve this issue.

So far there are no conclusive data with respect to the function of GDNF in the dorsal horn. Since both BDNF and GDNF containing fibers are concentrated in the superficial layers of the dorsal horn, which receive fibers that are primarily involved in nociceptive transmission, it seems likely that both substances are involved in nociception. If GDNF, as BDNF, would be localized in dense cored vesicles, this would imply the activity-dependent release of GDNF from primary afferent fibers and bring GDNF into the realm of neurotransmission. On the other hand, a recent study[10] showed that changes in the dorsal horn, that normally occur after a peripheral after nerve lesion, were partially reversed by intrathecal GDNF application, suggesting a trophic rather than a neuromodulator-like role for GDNF. The finding in the same study[10] that Ret is present in presumed terminals in the inner part of lamina II combined with our finding that GDNF is present in fibers terminating in lamina I and outer lamina II suggests that the subset of ganglion cells expressing GDNF does not express the Ret receptor. Again, similar observations have been made previously with respect to BDNF, which is expressed in a subset of ganglion cells, while another subset expressed Trk B, the BDNF receptor.[26]

CONCLUSION

The present study shows that GDNF is present in spinal ganglion cells and is transported anterogradely within primary afferent fibers, which terminate predominantly in the superficial dorsal horn. Since the superficial dorsal horn mainly receives nociceptive fibers, it seems likely that GDNF is involved in the processing of nociceptive input at the spinal level. Whether GDNF acts as an anterograde trophic messenger, maintaining normal neuronal functioning, or whether it has a more direct effect as a neuromodulator in pain transmission, remains to be elucidated.

Acknowledgements

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FIGURES

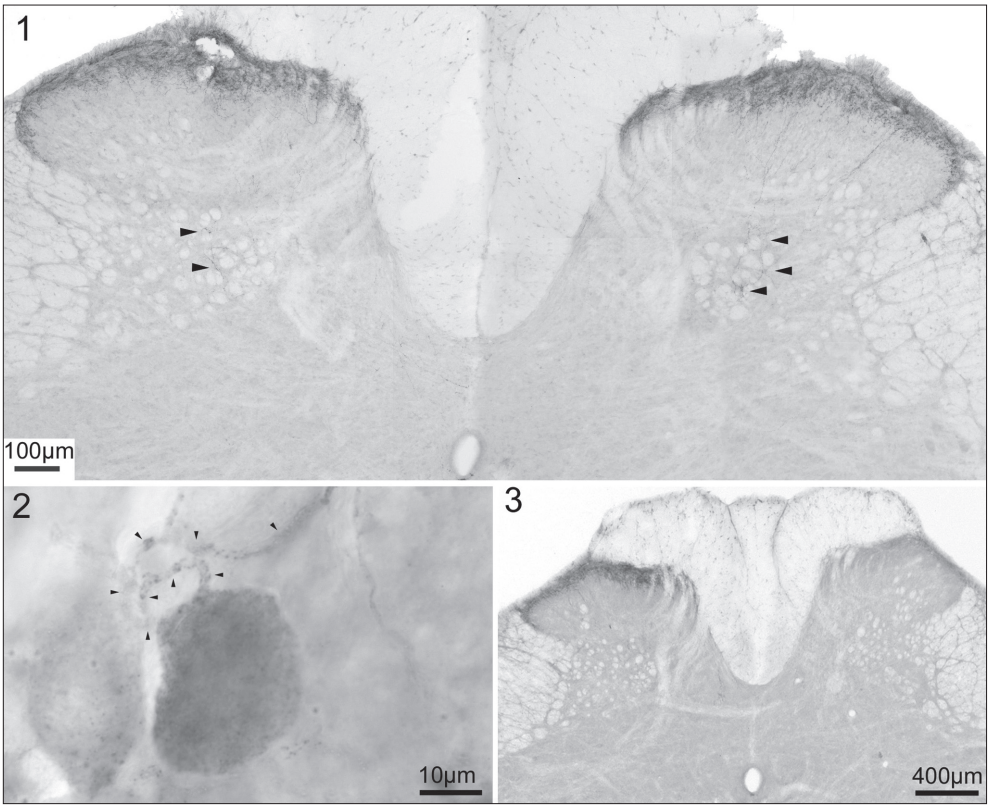

Fig. 1. Light micrograph of a section from the rat L5 segment showing dense GDNF immunoreactive fibers and presumed terminals in lamina I and outer lamina II. Several strongly immunoreactive fibers are also present in the central parts of laminae IV and V (arrowheads). The other laminae also contain a few varicose fibers.

Fig. 2. Light micrograph of a section from the L5 ganglion. A strongly GDNF-immunoreactive ganglion cell is present on the right. From this cell a curling fiber originates (arrowheads), that contains granular GDNF labeling. On the left, another, weakly labeled, ganglion cell is present. Both cells may contain a few immunoreactive granules.

Fig. 3. Light micrograph of a section from the L5 segment after a L3-S1 dorsal rhizotomy on the right side. Note the nearly complete disappearance of GDNF immunoreactivity on the rhizotomized side, while GDNF labeling on the non-rhizotomized control side is still present.

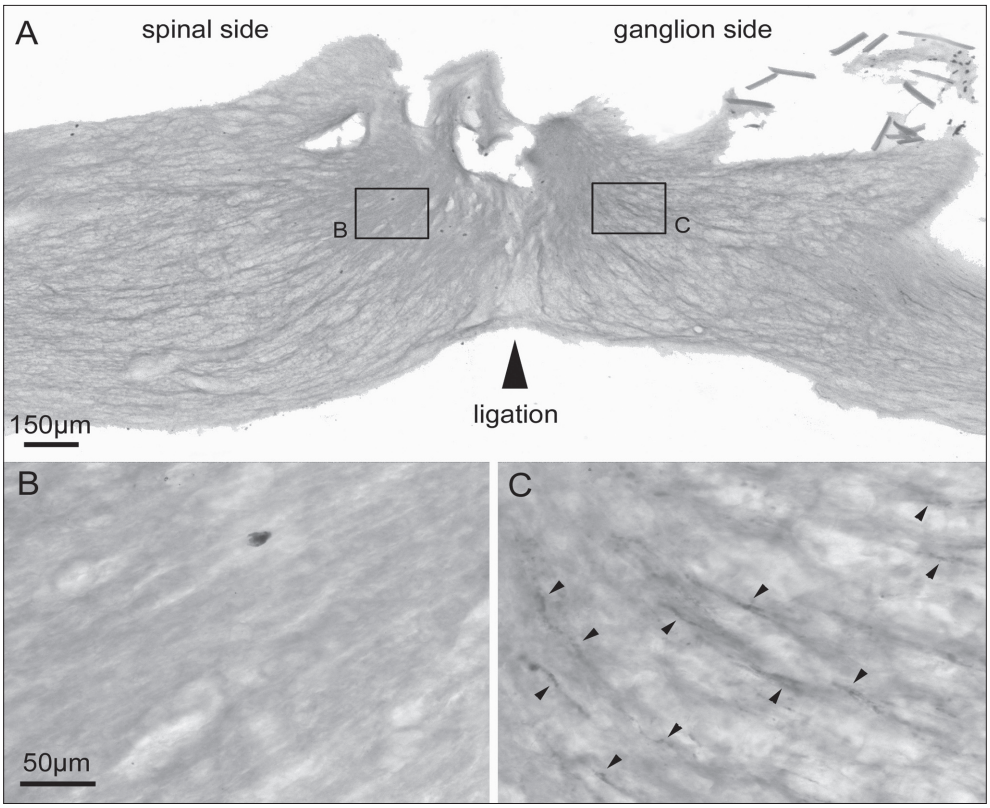

Fig. 4. (**a-c**) Light micrographs of a section through a gelatin embedded L5 dorsal root, 9 days after ligation. The section was treated for GDNF immunocytochemistry.

(**a**) Overview showing the area of ligation in the center (large arrowhead) with the spinal side on the left and the ganglion side on the right of the ligation. The boxes indicate the areas of magnification shown in (b) and (c).

(**b**) Magnification of the area indicated in (a) showing that GDNF immunoreactivity is absent on the spinal side of the ligation.

(**c**) Magnification of the area indicated in (a) showing GDNF immunoreactive fibers (small arrowheads) on the ganglion side of the ligation.

Chapter 3

Depletion of GDNF from primary afferents in adult rat dorsal horn following peripheral axotomy

Running title:
GDNF depletion from primary afferents after axotomy

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) is produced in a subset of adult rat spinal ganglion neurons and anterogradely transported to the superficial dorsal horn. In this study the effect of sciatic nerve axotomy on the expression of GDNF protein in the dorsal horn was investigated, using immunocytochemistry. Image analysis showed a 44% decrease relative to the non-transected side after 5 days survival, progressing to more than 80% decrease after 10 days and remaining so for at least 100 days. This rapid and strong decrease suggests active downregulation of the expression of GDNF protein after peripheral axotomy. The observed down-regulation of GDNF is compared with changes observed for other substances in primary afferents after peripheral axotomy and is discussed in light of its presumed trophic or transmitter role in nociception.

Key words: Axotomy; Dorsal horn; Glial cell line-derived neurotrophic factor; Image analysis; Immunocytochemistry; Neuropeptide; Rat; Spinal cord

INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) is a highly potent trophic factor for midbrain dopaminergic neurons, motoneurons and sensory neurons.[1-4] While distantly related to the transforming growth factor-β superfamily, GDNF is now considered as a member of a separate class of neurotrophic factors, together with neurturin and persephin.[5] GDNF mRNA is found in the adult neocortex, thalamus, hippocampus, cerebellum, mesencephalon, pons, medulla and spinal cord.[6] Using immunocytochemistry, we recently identified GDNF protein in dorsal root ganglion (DRG) neurons in the adult rat and demonstrated anterograde transport of GDNF within their central projections.[7] GDNF immunoreactivity (GDNF-IR) in the dorsal horn is derived exclusively from primary afferent fibres and is especially strong in laminae I and II-outer, suggesting that GDNF is preferentially localized in nociceptive fibres. A similar termination pattern was shown for a variety of other substances including brain derived neurotrophic factor (BDNF)[8] and neuropeptides like substance P (SubP), calcitonin gene-related peptide (CGRP), galanin (GAL) and vasoactive intestinal peptide (VIP).[9] While neuropeptides are known to be involved in the modulation of nociceptive transmission,[10] the role of BDNF and GDNF in the nociceptive system is still unclear.

Following peripheral axotomy a number of changes in the expression of neuropeptides in the dorsal horn has been observed, including downregulation of SubP and CGRP and up-regulation of GAL and VIP.[9] In addition, sciatic nerve axotomy may lead to up-regulation or de novo synthesis of BDNF immunoreactivity in presumed myelinated fibres in the ipsilateral gracile nucleus and lamina III and IV of the dorsal horn.[11] Thus, the expression of neuropeptides and BDNF is actively regulated in response to peripheral axotomy.

In this study, we investigated the effect of sciatic nerve axotomy on GDNF-IR in the superficial dorsal horn, in order to determine whether GDNF expression in primary afferents is also actively regulated in response to peripheral axotomy.

MATERIALS AND METHODS

Adult male Wistar rats were anesthetized with 2% halothane in O_2/N_2O (30/70%). The left sciatic nerve was exposed, dissected free and locally anesthetized with lidocaine. Subsequently, the nerve was transected and 5 mm was removed at the midthigh level to prevent regeneration of the proximal stump into the distal stump. The rats were allowed to survive for 5, 10, 25, 40 or 100 days ($n=3$ for each survival time), after which they received an overdose of sodium pentobarbital and were perfused as described previously.[7] Three normal rats were used as controls. After perfusion the lumbar spinal cord was dissected and from three experimental animals (10, 40 and 100 days survival) and one control animal, the medulla oblongata was also dissected. The right side was marked. Sections (34 μm) were cut on a freezing microtome and every fourth section

was collected. Then they were routinely processed for GDNF immunocytochemistry, using the ABC method and a specific antibody (Santacruz), as described previously.[7] Subsequently, the sections were randomly mounted on slides. For controls some sections were treated as above, except that the GDNF antibody was either substituted by saline or preincubated (2 h) with a 10-fold (by weight) excess of peptide antigen (Santacruz) in TBS.

For quantification of GDNF-IR, the first four L5 sections encountered on the slide were selected. These sections were photographed with a Kodak 64 ASA daylight film (diapositive) in a light microscope (Leica DMR) equipped with a photoautomat, using a 5x/0.12 objective and a CB 12 (blue) filter. After processing the diapositives were scanned at 337 dpi using a Polaroid SprintScan slide scanner connected to a Power Macintosh computer. The resulting images ($n=72$) were further analysed using the public domain NIH Image (v 1.61) program. A square selection of 128x128 pixels was made such that it contained the entire medial superficial dorsal horn on each side. Processing and image analysis of this selection was performed as described previously,[12] using a threshold of 75. This method created an image that appeared in black against a white background. This binary image was an accurate representation of the GDNF-IR area in the section and was expressed in number of pixels.

The mean pixel number of three animals was calculated for each side and for each survival time, using the mean of four sections per rat as one observation. The mean pixel number of the control sides of the experimental animals was compared with the right and left side of control animals, using the unpaired *t*-test. For each of the images from the experimental animals the difference between the pixel number of the control (right) side and the axotomized (left) side was expressed as a percentage of the control side. For the control animals the difference between the right and the left side was calculated in a similar way. The unpaired *t*-test was used to compare these percentages.

RESULTS

Light microscopic examination of the dorsal horn of sections from control rats showed strong GDNF-IR in the superficial dorsal horn of the L5 segment (lamina I and II-outer) and a few labeled fibres in the deeper layers (lamina II-inner, III, IV, V and X) on both sides (Fig. 1a, b), as described previously.[7] Control L5 sections incubated with saline or with GDNF antibody preincubated with peptide antigen did not show immunoreactive structures.

After axotomy, GDNF-IR on the axotomized side was strongly decreased, especially after longer survival times. The decrease occurred only in the medial dorsal horn, corresponding to the territory of the sciatic afferents, while immunoreactivity in the lateral part and on the contralateral side appeared as normal (Fig. 1c-g). Comparisons of the mean pixel numbers, representing the

area in the medial superficial dorsal horn occupied by GDNF-IR fibres, on the control side of experimental animals and either side of control animals, showed that they were not significantly different ($p>0.15$; Table 1). This allowed for a comparison between the control side and the axotomized side. The mean pixel number of the axotomized side showed a 44% decrease relative to the control side at 5 days after axotomy and between 82% and 91% at 10, 25, 40 or 100 days (Table 1). At all survival times the relative decrease in the experimental animals was significantly different from the relative difference between right and left in control animals (Table 1).

At 100 days after axotomy some GDNF-IR fibres reappeared in the medial superficial dorsal horn (Fig. 1g-h). Although these fibres were sparse, they were consistently present in all three animals that survived 100 days after axotomy.

GDNF-IR fibres were never observed in the dorsal column nuclei of the caudal medulla oblongata in the control animal nor in the three animals that survived 10, 40 or 100 days after axotomy.

DISCUSSION

Sciatic nerve axotomy resulted in a dramatic decrease in GDNF-IR in the medial superficial dorsal horn of the L5 segment on the axotomized side. Quantification of the immunoreactive area showed a rapid decrease of $> 80\%$ relative to the control side at 10 days, after which the decrease remained stable at this low level for at least 100 days.

The image analysis method employed in the present study represents an objective means for quantification of immunoreactive areas.[12] However, since the GDNF-IR area is susceptible to variations in the immunocytochemical procedure we used the control or right side as an internal control. This was justified, because there was no statistically significant difference in the analysed GDNF-IR area between the control side of the experimental animals and either side of the control animals. We therefore conclude that the reduction in the GDNF-IR area represents a decrease in GDNF protein, although the exact amount of this decrease cannot be determined with this technique.

Following peripheral axotomy, there is a rapid change in the expression of a number of substances within primary afferents that have a similar distribution as GDNF in the superficial dorsal horn. These changes include down-regulation of SubP and CGRP and up-regulation of GAL and VIP.[9,10] In contrast, BDNF immunoreactivity, which is also present in primary afferents of the superficial dorsal horn, is not clearly affected following peripheral axotomy (Holstege & Kennis, unpublished observations) and may even be upregulated or *de novo* expressed in myelinated primary afferents.[11] Thus, the rapid downregulation of GDNF after axotomy may represent a

similar phenomenon as observed for SubP and CGRP but is unlike that of GAL, VIP and BDNF, the only other neurotrophic factor now known to be present in primary afferent fibers.

We cannot rule out that the decrease of GDNF is secondary to a degenerative process rather than the result of down-regulation. Two recent studies[13,14] have shown selective degeneration of unmyelinated fibers, 4-8 months after sciatic nerve lesion. The rapid decline in GDNF-IR (> 80% at 10 days after axotomy), makes it unlikely that this decrease is secondary to degeneration of unmyelinated fibers. Peripheral axotomy also results in a series of progressing morphological changes of primary afferent terminals in the superficial dorsal horn, known as transganglionic degenerative atrophy (TDA).[15] Signs of TDA occur as soon as six days following peripheral axotomy[16] and therefore the decrease in GDNF-IR can also be explained as secondary to TDA. However, in that case all other substances within primary afferent fibers of the superficial dorsal horn would also be expected to decrease rapidly. In contrast, VIP, GAL and BDNF are upregulated or unaffected in the superficial dorsal horn after peripheral axotomy. We therefore conclude that the down-regulation of GDNF is an active process, induced by peripheral axotomy and is not secondary to primary afferent degeneration.

The GDNF-IR fibers that reappeared in the axotomized superficial dorsal horn at 100 days after axotomy may represent myelinated fibers expressing GDNF. This would fit with observations that peripheral nerve injury causes sprouting of myelinated afferents into the superficial dorsal horn,[17] some of which were reported to express BDNF.[11] However, the GDNF-IR processes could never be traced into laminae III and IV of the dorsal horn. Moreover, the gracile nuclei, which receive direct myelinated fibre input from lumbar dorsal ganglia, never showed any GDNF-IR neither in control nor in axotomized rats, including the one rat that survived 100 days after axotomy. It is therefore unlikely that the GDNF-IR fibers observed after 100 days survival represent myelinated fibers showing *de novo* expression of GDNF.

Studies on the functional role of GDNF in the sensory system (see Snider and McMahon[18] for a review) suggest that peripherally produced and retrogradely transported GDNF supports a subpopulation of nociceptors that are characterized by expressing GDNF receptor components and by their central projection to lamina II-inner.[19] Evidence for this trophic role of GDNF has been obtained from experiments[19] showing that intrathecal application of GDNF reversed the down-regulation of several substances in these GDNF-dependent nociceptors and prevented sprouting of myelinated fibers into the superficial dorsal horn. Our previous findings[7] have shown that a source of GDNF is actually present within primary afferents in lamina I and II-outer, which may provide the trophic supply for GDNF-dependent primary afferent nociceptors terminating in lamina II-inner. If this spinal source of GDNF is depleted after axotomy, as shown here, the lack of GDNF might induce the changes described above. It would also explain that intrathecally applied GDNF is able to counteract these changes, namely by substituting for the axotomy induced depletion of GDNF from lamina I and II-outer. If this hypothesis holds true, it would imply that GDNF, produced in

GDNF-independent DRG neurons, is anterogradely transported to the superficial dorsal horn to act as a trophic factor for GDNF-dependent DRG neurons.

Apart from its potential neurotrophic effects, GDNF may exert rapid effects on nociceptive transmission in the spinal cord, thus acting as a neuromodulator, as has been suggested for BDNF.[20] Support for this hypothesis is provided by preliminary data, [21] indicating that GDNF is at least partly present in dense cored vesicles in primary afferent terminals of the superficial dorsal horn, suggesting activity dependent release and that intrathecally applied GDNF rapidly increases c-Fos expression in the superficial dorsal horn, suggesting a rapid excitatory effect on spinal neurons. This is in line with the finding[22] that RET and GFRα-1, the receptor components necessary for GDNF signalling, are present in dorsal horn neurons, although this was not confirmed in another report.[23]

CONCLUSION

The dramatic decrease of GDNF-IR in the superficial dorsal horn after sciatic nerve section is most likely the result of active down-regulation of GDNF within primary afferents in response to peripheral axotomy. This behaviour resembles that of CGRP and SubP, which are both involved in nociceptive transmission, but is in contrast to that of GAL, VIP and BDNF, which are up-regulated or remain unchanged after axotomy. In order to clarify the functional meaning of the down-regulation of GDNF after axotomy, further study of its role in nociception, as a trophic factor and as a neuromodulator, is needed.

Acknowledgements

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FIGURES

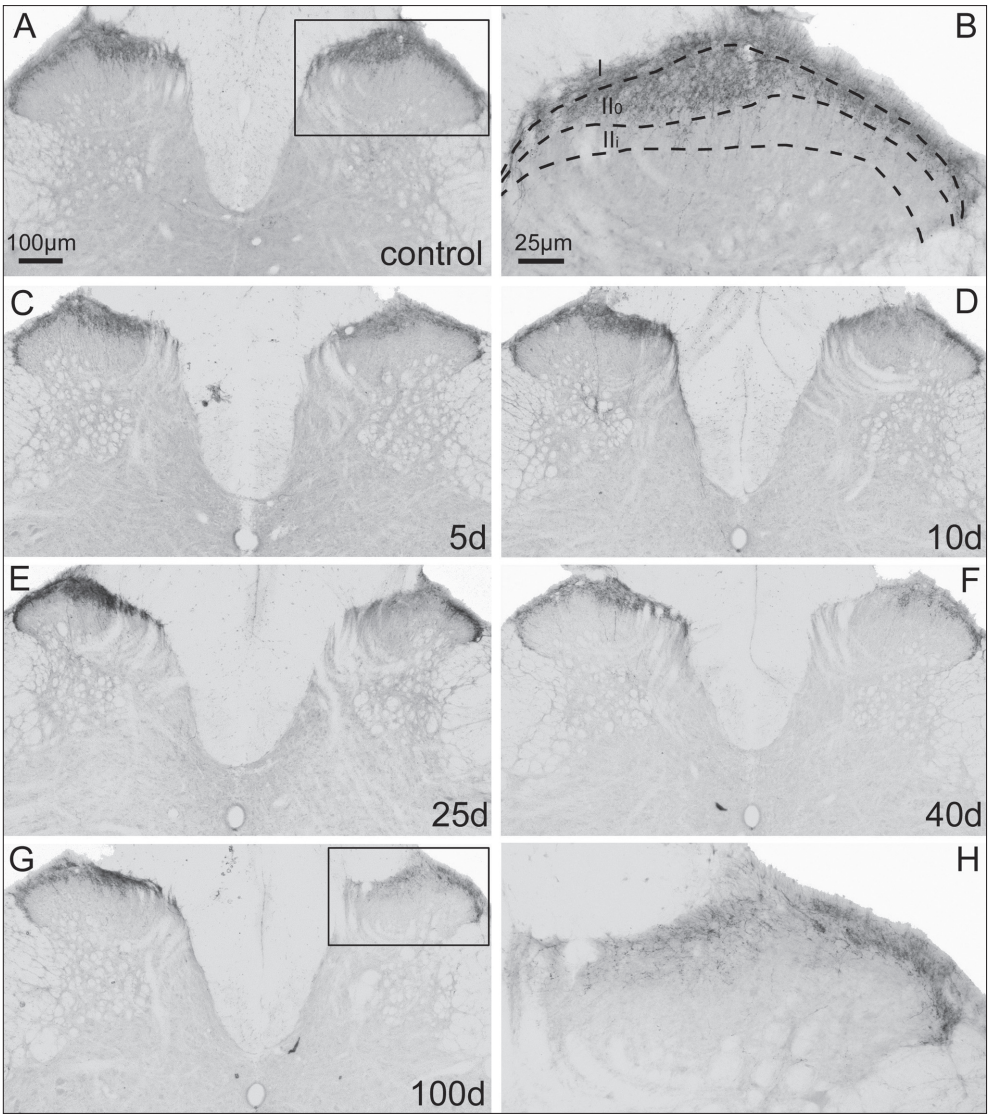

Fig. 1. Light micrographs of sections from the rat L5 segment, after processing for GDNF immunocytochemistry.

(**a**) Sections from a control animal, showing strong GDNF-IR in the superficial dorsal horn (lamina I and II-outer) and a few labeled fibers in the deeper layers (lamina II-inner, III, IV, V and X) on both sides. The box indicates the area of magnification shown in (b).

(**b**) Magnification of (a), showing that GDNF-IR is mainly present in the superficial dorsal horn. I is lamina I, IIo is the outer part of lamina II, IIi is the inner part of lamina II.

(**c-g**) Progressing decrease of GDNF-IR in the medial dorsal horn on the left (axotomized) side, while immunoreactivity on the lateral part and on the contralateral side remains intact. The box in (g) indicates the area of magnification shown in (h).

(**h**) Magnification of the axotomized dorsal horn 100 days after axotomy, showing three labeled fibers in the medial superficial dorsal horn

Table

	Control	5 days	10 days	25 days	40 days	100 days
Ipsilateral	1008	286	182	135	90	110
Contralateral	983	695	1054	965	809	930
% decrease	-5%	44%*	82%**	86%***	91%***	88%***

Values are mean pixel number of 3 animals per group.

Significant differences between controls and experimental animals: *P<0.05, **P<0.01, ***P<0.001, unpaired *t*-test

The areas of the control sides of experimental animals and either side of control animals were not significantly different ($p>0.15$, unpaired *t*-test)

Chapter 4

Intrathecal injection of GDNF and BDNF induces immediate early gene expression in rat spinal dorsal horn

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) are potent trophic factors for dorsal root ganglion cells. In addition, these factors are produced in subsets of dorsal root ganglion cells and transported anterogradely to their terminals in the superficial dorsal horn of the spinal cord, where they constitute the only source of GDNF and BDNF. We investigated the effect of 10 μg GDNF and BDNF injected by lumbar puncture, on the expression of the immediate early gene (IEG) products c-Fos, c-Jun and Krox-24 in the adult rat dorsal horn. In the dorsal horn of S1 spinal segments, GDNF and BDNF induced a strong increase in IEG expression, which was most pronounced in laminae I and II (2.9-4.5 fold). More distal from the injection site, in the dorsal horn of L1/L2 spinal segments, the increase in IEG expression was less pronounced, suggesting a concentration-dependent effect. In order to explain the effects of intrathecally injected GDNF, we investigated whether lumbo-sacral dorsal horn neurons expressed RET protein, the signal-transducing element of the receptor complex for GDNF. It was found that several of these neurons contained RET immunoreactivity and that some of the RET-labeled neurons had the appearance of nociceptive specific cells, confirming their presumed role in pain transmission. Additionally, using double-labeling immunofluorescence combined with confocal microscopy, it was found that after intrathecal GDNF injection 35% of c-Fos-labeled cells were also labeled for RET. These results demonstrate that intrathecally administered GDNF and BDNF induce IEG expression in dorsal horn neurons in the adult rat, supposedly by way of their cognate receptors, which are present on these neurons. We further suggest that the endogenous release of GDNF and BDNF, triggered by nociceptive stimuli, is involved in the induction of changes in spinal nociceptive transmission as in various pain states.

Key words: brain-derived neurotrophic factor, c-Fos, c-Jun, GDNF family receptor α1, glial cell line-derived neurotrophic factor, Krox-24, pain, neurturin, RET, tyrosine kinaseB

INTRODUCTION

Neurotrophic factors are traditionally considered to play a critical role in neuronal survival and differentiation during development and, during adulthood, in the maintenance of neuronal connections and regeneration of damaged neurons (Barde, 1989; Lewin and Barde, 1996). For these purposes, neurotrophic factors are taken up by neurons in their target area through high-affinity receptors and retrogradely transported to their cell bodies. However, more recent findings showed that brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), which are both members of the neurotrophin family, are produced within neurons and transported in an anterograde rather than in a retrograde direction (for reviews see Altar and DiStefano, 1998; Conner et al., 1998; von Bartheld et al., 2001). Evidence for anterograde transport of BDNF was first obtained in central and peripheral projections of dorsal root ganglion (DRG) neurons (Zhou and Rush, 1996) and subsequently in the central nervous system (Altar et al., 1997). Glial cell line-derived neurotrophic factor (GDNF), a member of the GDNF family of proteins and unrelated to the neurotrophins (Lin et al., 1993; Airaksinen and Saarma, 2002), is also transported in an anterograde direction within primary afferent fibers (Holstege et al., 1998; Ohta et al., 2001; Rind and von Bartheld, 2002). Ultrastructural data suggest that anterogradely transported BDNF and GDNF are stored in dense-cored vesicles in primary afferent terminals in lamina I and II-outer of the dorsal horn (Michael et al., 1997; Holstege et al., 1999; Ohta et al., 2001). Since this region of the dorsal horn receives mainly nociceptive afferent input, it seems likely that both BDNF and GDNF are involved in the processing of nociceptive information in the spinal dorsal horn. Indeed, BDNF has been shown to modulate pain transmission in the spinal cord, especially by inducing hypersensitivity in pathological pain states (Kerr et al., 1999; Mannion et al., 1999). This effect is likely mediated by the receptor for BDNF, tyrosine kinase B (TrkB), which is expressed on spinal dorsal horn neurons (Yan et al., 1997a; Mannion et al., 1999; Garraway et al., 2003). These findings, together with data that BDNF can directly excite cells through TrkB receptors (Kafitz et al., 1999), strongly suggest that BDNF acts as a neuromodulator in spinal pain transmission (Pezet et al., 2002).

With regard to GDNF, previous studies (Bennett et al., 1998; Boucher et al., 2000; Ramer et al., 2000; Paveliev et al., 2004) have shown that it exerts various trophic and regenerative effects on adult primary afferent neurons. GDNF acts on neurons that express the receptor components for GDNF signaling: the signal transducing element RET and a ligand binding domain, known as GDNF family receptor $\alpha 1$ (GFR$\alpha 1$) (Airaksinen and Saarma, 2002; Tsui-Pierchala et al., 2002). Recently it was shown that the neural cell adhesion molecule NCAM can also function as a signaling receptor for GDNF (Paratcha et al., 2003). In addition to the long-term effects described in the peripheral nervous system and spinal cord, there are reports showing acute effects of GDNF on synaptic transmission in other parts of the central nervous system (Ribchester et al., 1998; Yang et al., 2001; Wang et al., 2003). Evidence for acute effects of GDNF combined with the

anterograde transport of GDNF to the superficial dorsal horn and its localization in dense-cored vesicles suggests that GDNF, like BDNF, acts as a neuromodulator in spinal pain transmission.

The main goal of the present paper was to determine whether intrathecal injection of GDNF and BDNF induces a rapid activation of dorsal horn neurons, as indicated by increased expression of the immediate early gene (IEG) products c-Fos, c-Jun and Krox-24 (Hunt et al., 1987). We also studied IEG expression after intrathecal injection of neurturin (NRTN), another neurotrophic factor of the GDNF family, although the evidence that NRTN is involved in pain transmission is limited (Vellani et al., 2004). In order to explain increased IEG expression after intrathecal injection of a neurotrophic factor, the presence of its cognate receptor is critically important. Since the presence of RET mRNA has not been unequivocally demonstrated in adult rat dorsal horn neurons, we additionally studied RET protein expression in the rat spinal cord using immunohistochemistry (IHC). Finally, we studied c-Fos and RET double-labeling after intrathecal GDNF injections, using immunofluorescence (IF) combined with confocal microscopy. Increased expression of IEGs after intrathecal GDNF and BDNF injection, combined with our previous observation that these trophic factors are present in dense-cored vesicles in primary afferent terminals in the dorsal horn, would strengthen the idea that GDNF and BDNF act as modulators of spinal pain transmission.

MATERIALS AND METHODS

Intrathecal injections and perfusion

24 Adult male Wistar rats were successfully injected and processed in five separate sessions. The first two sessions each consisted of one animal injected with vehicle, one with GDNF, one with BDNF and one with N-methyl-d-aspartate (NMDA). Session three and four each consisted of 5 animals, by including one animal with neurturin (NRTN). The last session consisted of two animals injected with vehicle, two with NRTN and two with GDNF. The injection procedure was as follows: rats were anaesthetized with 2% halothane in O_2/N_2O (30/70%) and shaved at the lower back. A lumbar puncture was performed (Sandkühler et al., 1996) between the L4-L5 or L5-L6 vertebrae, using a 30-gauge needle through which 40 μl of vehicle alone (1% bovine serum albumin in 0.025M phosphate buffer), mixed with 3 μl 10% Alcian Blue (an inert dye), or vehicle and Alcian Blue also containing 10 μg human recombinant NRTN (PeproTech Inc., Rocky Hill, NJ), 10 μg rat recombinant GDNF (R&D systems, Minneapolis, MN), 10 μg human recombinant BDNF (kindly donated by Regeneron, Tarrytown, NY) or 25 nmol NMDA (Sigma, St. Louis, MO). Alcian Blue was added to the injection fluid, in order to determine after dissection whether the injection fluid had actually been delivered intrathecally. Immediately after injection the animals were replaced in their cages. After 90 minutes they received an overdose of sodium pentobarbital

and were perfused transcardially with 250 ml of 0.025 M phosphate buffered saline (PBS) containing 0.8% sucrose and 0.4% D-glucose, followed by 750 ml of 4% paraformaldehyde in 0.05 M phosphate buffer (PB) (20^{0}C), the last 375 ml of fixation fluid containing 15% sucrose for cryoprotection. The spinal cord was exposed and inspected for spread of the injection fluid (i.e. the Alcian Blue). Animals with extradural injection, lesioned spinal roots or a lesioned conus medullaris were discarded. Then, the lumbo-sacral spinal cords of the successfully injected animals were dissected. All experiments had been approved by the Rotterdam Animal Ethics Committee.

Immunohistochemistry

For *IEG immunohistochemistry*, all spinal cords from one session were processed simultaneously as follows: they were postfixed for two hours at 20^{0}C in the fixation fluid containing 15% sucrose, transferred to 30% sucrose in 0.05M PB and left overnight at 4^{0}C. Sections (40 μm) were cut on a freezing microtome and collected for IHC. They were preincubated (1h at 20^{0}C) with 10% normal goat serum (NGS) and 0.3% Triton X-100 in 0.05M PBS, followed by incubation (60 h at 4^{0}C) with the primary antibodies, 1% NGS and 0.1% Triton in PBS. Primary antibodies were rabbit anti-c-Fos (1:40,000; Oncogene Research Products, La Jolla, CA), rabbit anti-c-Jun/AP-1 (1:20,000; Oncogene) and rabbit anti-Egr-1/Krox-24 (1:10,000; SantaCruz Biotechnology, SantaCruz, CA). Subsequently, sections were rinsed and incubated (1.5h at 20^{0}C) with biotinylated goat-anti-rabbit (1:200; Vector Laboratories Inc., Burlingame, CA), 1% NGS and 0.1% Triton in PBS. Finally, they were rinsed and placed in ABC reagent (avidin/biotin/peroxidase, Vector) containing 0.3% Triton (1.5h at 20^{0}C), rinsed again with 0.05M PB and reacted with 0.05% 3'-3 diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 dissolved in PB. Sections from each animal and each antibody were randomly mounted on a slide and coverslipped, using Permount (Fisher, Hampton, NH) as mounting medium.

BDNF immunohistochemistry was performed on 2 rats that had undergone intrathecal injection with 4 and 0.7 μg BDNF respectively. The BDNF antibody used here is a polyclonal antibody produced in rabbits and raised against *E. coli*-derived recombinant human BDNF (1:1500; kindly donated by Dr. Q. Yan, Amgen Inc., Thousand Oaks, CA). For specificity of the antibody see (Yan et al., 1997b). Perfusion and IHC were performed according to (Holstege et al., 1997), but without tyramide signal amplification.

RET immunohistochemistry was performed on 6 naive rats. The RET antibody used here is a biotinylated polyclonal antibody produced in goats and raised against recombinant mouse RET extracellular domain (1:100; R&D systems, Minneapolis, MN). The antibody was tested with the two isoforms that exist and was found to identify both isoforms, in line with the fact that the antibody is directed against the common extracellular domain of RET (de Graaff et al., 2001). The

same perfusion protocol as for IEG IHC was used, except that 0.25% glutaraldehyde was added to the fixation fluid and sections were postfixed for 2 hours at 20°C and overnight at 4°C. The same immunohistochemical protocol as for IEG IHC was used except that sections were heated in 25mM sodium citrate for 30 minutes at 80°C (Jiao et al., 1999) prior to incubation with normal serum, and 0.1M Tris buffered saline was used instead of PBS. Controls included omitting the primary RET antibody and preabsorbing with the synthetic protein the antibody was raised against (R&D systems).

For *c-Fos and RET double-labeling immunofluorescence*, the same perfusion and immunohistochemical protocol as for IEG IHC was used, c-Fos and RET antibody were diluted 1:5000 and 1:40 respectively. Cy3-conjugated donkey anti-goat and FITC-conjugated donkey anti-rabbit were used as secondary antibodies (1:200, 1.5h at 20°C; Jackson ImmunoResearch, West Grove, PA). Vectashield (Vector) was used as mounting medium.

Sections processed for BDNF, IEG and RET IHC were photographed using a Leica DC 300 digital camera on a Leica DMRB light microscope. Sections processed for c-Fos and RET double-labeling IF were photographed using a Zeiss LSM 510 confocal laser scanning microscope. Figures were composed using Adobe Photoshop 7 (Adobe Systems Inc., San Jose, CA). Adjustments were made only to brightness/contrast and sharpness.

Collection and analysis of data from the intrathecal injection experiment

For *cell counts and analysis of IEG immunohistochemistry*, the slides were blinded and analyzed with a camera lucida microscope (Neurolucida, MicroBrightfield Inc., Williston, VT). For each animal and each antibody (anti-c-Fos, -c-Jun, -Krox-24), the first four S1-sections encountered on a slide were selected. For animals injected in session one to four, also the first four L1/L2-sections encountered on a slide were selected. The S1-segment was defined primarily by the size and shape of the dorsal horn and dorsal columns and on this basis distinguished from the S2-segment. Sections containing the dorsomedial or dorsolateral motor nuclei (Holstege et al., 1996) were considered belonging to the L6-segment and discarded from analysis. The L1/L2-segment was defined by the size and shape of the ventral horn and on this basis distinguished from T13 and L3-segments (Molander et al., 1984). The outline of the dorsal horns and boundaries between laminae were identified bilaterally (Molander et al., 1984) and lines were drawn accordingly, using a camera lucida microscope. IEG-immunoreactive (-ir) nuclei were plotted in these drawings. Cells were counted separately in laminae I and II, in laminae III and IV and in lamina V. After all sections had been counted, the slides were deblinded and results were quantitatively assessed. Mean cell counts of four sections per animal were considered as one observation. Data were presented as means ± SEM of relative increase of IEG-ir cells, using the following formula: relative increase of cells $= x_i / \bar{x}_v$, where x_i is the absolute number of cells after injection of vehicle, NRTN,

GDNF, BDNF or NMDA and $\bar{x}_v$ is the mean number of cells after vehicle injection. The results of vehicle (n=6 at S1, n=4 at L1/L2), NRTN (n=4 at S1, not determined at L1/L2), GDNF (n=6 at S1, n=4 at L1/L2), BDNF (n=4 at S1 and L1/L2) and NMDA (n=4 at S1 and L1/L2) treated animals were compared using the unpaired t-test in laminae I and II (where cell counts were normally distributed) or Mann-Whitney's rank-sum test in laminae III and IV and lamina V (where cell counts were non-normally distributed), for each antibody separately (Stata 7.0; Stata Corporation, College Station, TX).

To analyze the degree of *c-Fos and RET double-labeling*, we used double-labeling IF combined with confocal microscopy on S1 sections adjacent to the sections used for immunoperoxidase labeling of IEGs, from three animals that had received intrathecal injections of 10 μg GDNF. The lateral half of lamina I and II from those sections, where the large majority of c-Fos-labeled cells was present, was systematically scanned for c-Fos-ir cells, RET-ir neurons and double-labeled neurons, using a Zeiss LSM 510 confocal laser scanning microscope and a 20x objective. The percentages of double-labeled neurons as a proportion of total number of c-Fos-ir cells and RET-ir neurons were expressed as means ± SEM, using Stata 8.0.

RESULTS

Expression and quantification of IEGs in the spinal dorsal horn after intrathecal injections of neurotrophic factors

After perfusion of the intrathecally-injected rats, the successful injections were identified by the staining due to the Alcian Blue in the injection fluid. Blue staining was present around the lumbo-sacral roots and the caudal part of the spinal cord. More rostrally, the blue staining slowly faded but always reached low thoracic segments, indicating that the injected fluid had diffused intrathecally alongside at least the entire lumbo-sacral cord (see also Sandkühler et al., 1996 and Kusmirek et al., 1997). In a separate experiment we used BDNF IHC to study diffusion of intrathecally injected BDNF. Exogenous BDNF was detectable from S2 up to T10 as a rim of immunoreactive product around the entire spinal cord including the dorsal horn, where also endogenous BDNF immunoreactivity was present in the superficial layers (see also Zhou and Rush, 1996). Density and thickness of exogenous BDNF immunoreactivity gradually decreased in a caudo-rostral direction and was dependent on the amount of intrathecally injected BDNF (Fig.1).

Light microscopical examination of the sections processed for c-Fos, c-Jun or Krox-24 IHC showed almost exclusively nuclear staining, which could be easily identified (Fig. 2). After intrathecal injection of *vehicle*, a few c-Fos-ir and c-Jun-ir cells were scattered throughout the dorsal horn, both in S1-sections and in L1/L2-sections. Krox-24-ir cells after *vehicle* injection

were observed mainly in lamina III and IV (see also Herdegen et al., 1991). After *NRTN* injection, the distribution of c-Fos, c-Jun and Krox-24-ir cells at the S1 spinal level was similar as observed after vehicle injection and the number of IEG-ir cells after *NRTN* injection was not significantly different from that after vehicle injection ($p>0.05$; Fig. 3, Table).

After intrathecal *GDNF* injection, c-Fos, c-Jun and Krox-24 expression in laminae I and II at the S1 spinal level was significantly increased compared to both vehicle and NRTN ($p\leq0.01$). At the L1/L2 spinal level, c-Fos and c-Jun expression ($p\leq0.05$), but not Krox-24 expression ($p=0.07$), was significantly increased compared to vehicle in laminae I and II. The relative increase at S1 (2.9-4.5 fold) was greater than at L1/L2 (2.3-2.4 fold). In laminae III and IV and in lamina V c-Jun expression was never increased, while c-Fos expression was significantly increased in laminae III and IV at S1 and at L1/L2 and Krox-24 expression was significantly increased in laminae III and IV and in lamina V at S1 ($p\leq0.05$, all compared to vehicle).

After intrathecal *BDNF* injection, IEG expression was significantly increased in laminae I and II at S1 ($p\leq0.05$ compared to vehicle and NRTN), while at L1/L2 the increase was not statistically significant. In laminae III and IV and in lamina V, there was no statistically significant increase.

IEG expression in laminae I and II after GDNF and BDNF injection was not statistically different from IEG expression after injection of *NMDA*, both at S1 and at L1/L2 ($p>0.05$).

Description of immunohistochemical expression of RET in the dorsal horn of naïve rats

Light microscopical examination of S1 sections from naïve rats processed for RET IHC showed a dense band of presumed terminal labeling in lamina II-inner. In the ventral horn labeling of motoneurons was observed. In addition, several strongly RET-ir neurons (Fig. 4) and a significant number of weakly labeled neurons were observed scattered in lamina I and II, as well as in the deeper layers of the dorsal horn. In neuronal somata RET labeling was prominent in the cytoplasm and sometimes extended into the primary dendrites. Typically most sections showed a few RET-ir cells in lamina I, which appeared as fusiform neurons, possibly representing nociceptive specific neurons (Lima and Coimbra, 1983; Han et al., 1998). Immunoreactivity was absent in sections in which the primary antibody was omitted or preincubated with the synthetic protein the antibody was raised against.

Expression and quantification of c-Fos and RET double-labeling

The general labeling pattern of c-Fos and RET obtained with IF and confocal microscopical imaging, was the same as described for light microscopy. Double-labeled neurons were characterized by nuclear labeling for c-Fos with sparing of the nucleolus, surrounded by cytoplasmatic labeling for RET which sometimes extended into the primary dendrites (Fig. 5). A total number of 251 immunoreactive cells were counted in the lateral superficial dorsal horn of S1 sections from three GDNF injected animals. It was found that 35 ± 2% (mean ± SEM) of c-Fos-labeled cells were also labeled for RET and 40 ± 4% (mean ± SEM) of RET-labeled neurons were also labeled for c-Fos.

DISCUSSION

IEG induction by intrathecal administration of GDNF and BDNF

We have shown here that intrathecal injection of GDNF or BDNF through lumbar puncture induces a strong increase in the number of neurons expressing c-Fos, c-Jun and Krox-24 in the dorsal horn of the adult rat S1 spinal segment. The increase in IEG expression is most pronounced in the superficial dorsal horn, i.e. lamina I and II, and of the same magnitude as the increase after intrathecal NMDA injection. Intrathecal injection through lumbar puncture is a minimally invasive technique, which has no confounding effects on spinal nociception and does not induce c-Fos expression in the spinal dorsal horn (Sandkühler et al., 1996). Accordingly, we found that only a few cells express IEGs after vehicle injection, as in untreated rats. Furthermore, after injection of NRTN, a member of the GDNF family (Kotzbauer et al., 1996; Airaksinen and Saarma, 2002), the number of cells expressing IEGs remained low as in vehicle treated rats. Based on the above it is concluded that the increase in IEG expression that we observed after GDNF and BDNF injection is the direct result of the presence of these substances in the injection fluid.

The finding that NRTN, in contrast to GDNF and BDNF, did not induce IEG expression, may be regarded as somewhat surprising, given that the receptor components for NRTN, i.e. GFRα2 (Widenfalk et al., 2001) and RET, are present in the adult rat dorsal horn. To our knowledge there is only one other paper studying the *in vivo* effects of NRTN on nociceptive transmission (Hoane et al., 1999). In this paper an allodynic effect of intraventricularly administered GDNF is described, while no such effect was detectable for NRTN. Since the recombinant NRTN protein that we used has been shown to be biologically active (e.g. Coulpier et al., 2002 and Holm et al., 2002), one may speculate that NRTN does not penetrate as deeply as GDNF into nervous tissue, as also suggested by others (Hoane et al., 1999; Rosenblad et al., 1999). Alternatively, the effect of NRTN on dorsal horn neurons may not include activation of intracellular pathways that lead to increased expression of the IEGs that we have investigated.

We found that the IEG changes in the L1/L2 segments are less pronounced than in the S1 segment. This is likely a concentration-dependent effect, since the spread of intrathecally injected substances is generally limited (Sandkühler et al., 1996; Kusmirek et al., 1997), leading to a rapid decrease in concentration at relatively limited distances from the injection site. Accordingly, the intensity of Alcian Blue staining of the spinal cord was weaker at high lumbar levels, i.e. further away from the injection site, than at high sacral levels. In line with these findings immunoreactivity of intrathecally administered BDNF decreased from S2 to T10. Furthermore a lower dose of BDNF caused an overall weaker immunohistochemical staining. It seems likely that the same effects hold true for the other substances that we administered. Thus, the lower level of IEG expression at high lumbar as compared to high sacral levels probably reflects a lower concentration of the injected substances at these high lumbar levels.

Expression of RET in spinal dorsal horn neurons

The distribution of RET-immunoreactivity in the spinal cord is in agreement with previous reports showing localization in primary afferent terminals in lamina II-inner and in motoneurons in the spinal cord (Bennett et al., 1998; Duberley et al., 1998). We also observed several strongly RET-ir neurons in both the superficial and deep layers of the dorsal horn. Additionally, a substantial number of weakly RET-ir neurons was observed. This may be due to low expression levels of RET in these neurons, which makes it difficult to detect them by IHC and which may also explain why RET mRNA has so far not been unequivocally demonstrated in the adult rat dorsal horn (Glazner et al., 1998; Widenfalk et al., 2001). The presence of RET protein in dorsal horn neurons, shown here for the first time, is a prerequisite to explain the induction of the IEGs after intrathecal administration of GDNF. At the subcellular level it was observed that RET immunoreactivity in dorsal horn neurons was preferentially localized in the cytoplasm, which is in line with descriptions of neuronal RET-immunoreactivity elsewhere in the nervous system (Arce et al., 1998; Bennett et al., 1998; Walker et al., 1998).

How do GDNF and BDNF act on dorsal horn neurons?

Several studies have shown direct facilitatory effects of locally administered BDNF on spinal pain transmission (Kerr et al., 1999; Mannion et al., 1999). These effects are considered to be mediated by TrkB receptors, which are expressed on spinal dorsal horn neurons (Yan et al., 1997a; Mannion et al., 1999). In a recent study using spinal cord slices, a direct action of BDNF on substantia gelatinosa cells was identified leading to prolonged facilitation of dorsal horn neurons (Garraway et al., 2003). Although some primary afferents that terminate in the spinal dorsal horn express TrkB, it is unlikely that these presynaptic receptors are involved in the effects of BDNF on nociception, since these fibers are non-nociceptive in nature and terminate preferentially in the

deep dorsal horn (Michael et al., 1997; Carroll et al., 1998). Therefore the effect of BDNF on spinal pain transmission is likely mediated by postsynaptic TrkB receptors on dorsal horn neurons, as observed elsewhere in the brain (Kovalchuk et al., 2002; Manabe, 2002).

GDNF preferentially acts through GFRα1 receptors, using RET as the signal-transducing element. Both GFRα1 and RET are expressed in primary afferent neurons (Bennett et al., 1998) and we now show that RET, in addition to GFRα1 (Glazner et al., 1998; Matsuo et al., 2000; Widenfalk et al., 2001), is also expressed in adult rat dorsal horn neurons. It seems unlikely that GDNF induces IEG expression by acting presynaptically on primary afferent fibers, since the distribution of IEG-ir neurons after GDNF injection does not match the known distribution of RET-ir primary afferent terminals, which are present primarily in lamina II-inner of the dorsal horn. Since several strongly RET-ir neurons and a significant number of weakly labeled neurons were identified in both the superficial and deep dorsal horn and since 35% of c-Fos-labeled cells were also labeled for RET in the superficial dorsal horn, we suggest that intrathecally injected GDNF induces IEG expression in the dorsal horn at least partly through RET receptors on dorsal horn neurons. The observation that not all c-Fos-labeled cells were also RET-labeled may be due to limitations of immunohistochemical detection of RET, or may indicate that other receptors, like NCAM, are involved in GDNF signaling in the spinal dorsal horn. NCAM is diffusely expressed in both the adult chick and mouse spinal gray matter (Daniloff et al., 1986; Brook et al., 2000) and its polysialylated form (PSA-NCAM) is expressed mainly in fibers but also occasionally in neurons of the adult rat superficial dorsal horn (Bonfanti et al., 1992; Seki and Arai, 1993; Bonfanti et al., 1996). Although the possibility that GDNF participates in the regulation of synaptic plasticity through activation of NCAM has been suggested to occur in the brain (Paratcha et al., 2003), there is presently no evidence for such interactions in the spinal cord.

Taken together, the above findings suggest that both GDNF and BDNF induce IEG expression in dorsal horn neurons at least partly through direct activation of their postsynaptic tyrosine kinase receptors.

GDNF and BDNF as modulators of spinal pain transmission

c-Fos, c-Jun and Krox-24 expression in dorsal horn neurons increases after noxious, but not after non-noxious stimulation (Hunt et al., 1987; Herdegen et al., 1991). Especially after GDNF administration, we found increased expression of c-Fos and Krox-24 in the superficial and deep dorsal horn, while the increase in c-Jun expression was restricted to the superficial dorsal horn, a similar distribution as seen after electrical stimulation of nociceptive fibers (Herdegen et al., 1991). These findings suggest that IEG upregulation after intrathecal injection of GDNF and BDNF preferentially occurs in neurons that receive input from nociceptive primary afferents. Accordingly, some of the RET-ir neurons in the superficial dorsal horn had the typical appearance of fusiform

neurons which, based on their morphology, are known to be nociceptive specific (Han et al., 1998), indicating that RET expressing cells in the superficial dorsal horn are indeed involved in nociceptive transmission.

The region where the strongest IEG upregulation was observed after intrathecal injection of GDNF and BDNF, i.e. the superficial dorsal horn, coincides with the area in which GDNF-ir and BDNF-ir primary afferent terminals are present. This leads to the hypothesis that a similar IEG upregulation as described here, may also occur following release of endogenous GDNF and BDNF from nociceptive primary afferent terminals, which constitute the only source of endogenous GDNF and BDNF in the dorsal horn (Zhou and Rush, 1996; Holstege et al., 1998). Unlike BDNF, GDNF mRNA has so far not been detected in DRG neurons, possibly because mRNA levels are too low to be detected by in situ hybridization (compare Stöver et al., 2001). GDNF protein levels in the somata of DRG neurons are also low (Bar et al., 1998; Holstege et al., 1998; Ohta et al., 2001). This may be due to low production or may indicate that GDNF protein is directly transported to the terminals in the dorsal horn, where it accumulates and is detected more easily. Alternatively, GDNF protein may be taken up in the periphery and transported transganglionically (Ohta et al., 2001; Rind and von Bartheld, 2002; von Bartheld, 2004).

Release of BDNF in the spinal cord from primary afferent fibers in response to nociceptive stimuli leads to increased postsynaptic excitability and behavioral effects suggesting that BDNF is involved in the induction of central sensitization (Pezet et al., 2002). C-Fos, and possibly other IEGs, are involved in downstream events following activation of TrkB by BDNF and their increase may thus reflect the central changes in nociception that develop after intrathecal BDNF injection (Herdegen and Leah, 1998; Kerr et al., 1999; Pezet et al., 2002). The present finding that intrathecal GDNF, like BDNF, induces an increase in IEG expression, may indicate that also GDNF released from nociceptive fibers in the spinal cord is directly involved in spinal nociceptive processing. Although this idea is supported by increasing evidence obtained in other neuronal systems that GDNF has acute effects on excitability (Ribchester et al., 1998; Yang et al., 2001; Wang et al., 2003), there are conflicting data about its role in nociceptive transmission. One report describes allodynia following intraventricular administration of 1.25 μg/h GDNF (Hoane et al., 1999), which findings are supported by data showing an anti-hyperalgesic effect of an intrathecally administered GDNF function-blocking antibody in complete Freund's adjuvans induced inflammation (Fang et al., 2003). However, Boucher et al., 2000, who showed that intrathecal administration of 0.5 μg/h GDNF had potent analgesic effects in rat models of neuropathic pain, also described that the same dosage did not affect mechanical and thermal thresholds in normal animals. Although differences in GDNF concentration and type of administration could account for these seemingly contradictory observations, it remains difficult to relate the increase in IEG expression that we found to a specific change in pain behavior. Obviously detailed behavioral tests need to be carried out in conjunction with IEG IHC to learn more about the exact role of GDNF and IEGs in pain transmission.

In this study we have shown that intrathecal injection of GDNF and BDNF induces a strong increase of three different IEGs in dorsal horn neurons, supposedly through their cognate receptors which are present on these neurons. Similarly, release of endogenous GDNF and BDNF, triggered by nociceptive input, may lead to increased IEG expression in dorsal horn neurons and subsequently to changes in spinal nociceptive transmission, like central sensitization, in the adult rat. Thus, in addition to their trophic effects on developing and regenerating neurons, we suggest that both GDNF and BDNF act as neuromodulators in the adult spinal dorsal horn. As a consequence GDNF and BDNF may be considered as multifunctional signaling proteins that are especially important in the nociceptive system. Modulation of the synthesis and release of these factors in various painful conditions may eventually prove an effective tool for alleviating pain.

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FIGURES

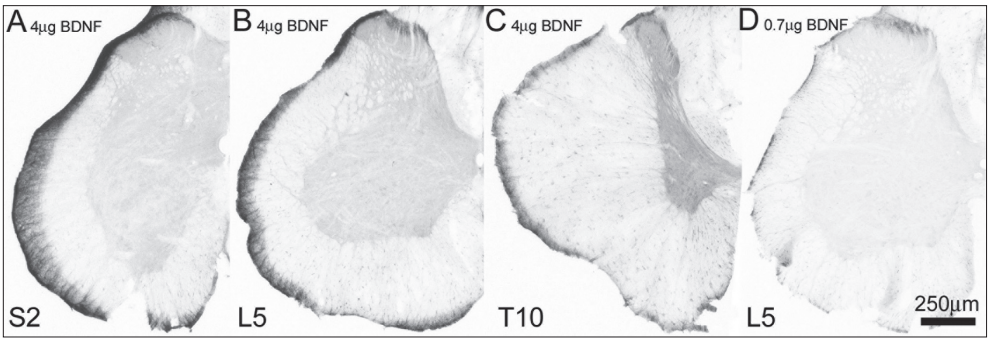
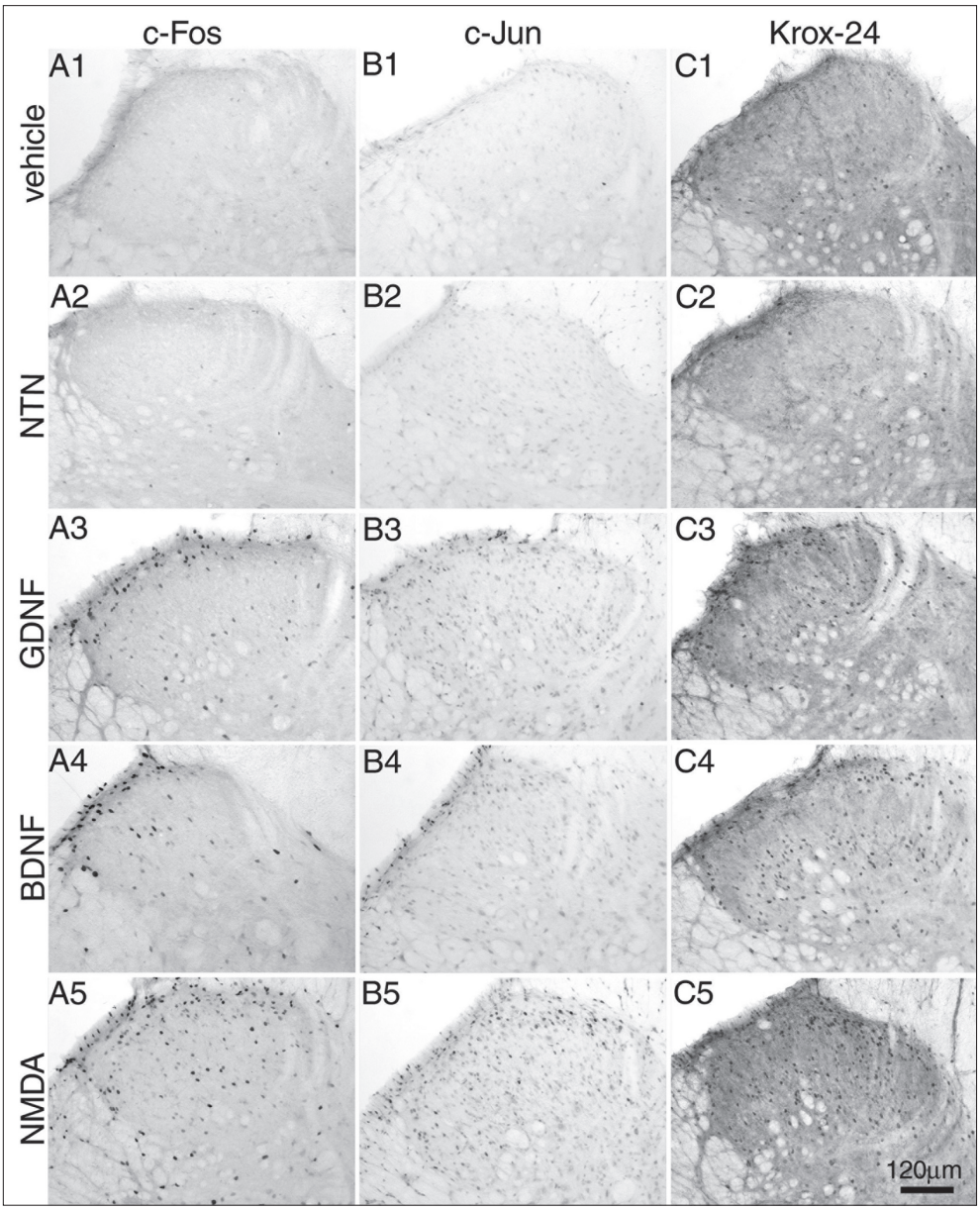


Fig.1. Light micrographs, showing rat spinal cord sections after intrathecal injection of BDNF, followed by processing for BDNF IHC. In S2 (A) and L5 (B) sections, exogenous BDNF-immunoreactivity can be seen overlapping with endogenous BDNF-immunoreactivity in the superficial dorsal horn. More rostral from the injection site, exogenous BDNF-immunoreactivity is decreased, but could still be immunohistochemically detected in T10 (C) sections. Injection of 0.7 µg of BDNF resulted in weaker BDNF labeling in L5 sections (D) as compared to 4 µg of BDNF.

Fig. 2. (next page) Light micrographs, showing sections from the rat S1 segment after intrathecal injection with vehicle, NRTN, GDNF, BDNF or NMDA, followed by processing for c-Fos (A), c-Jun (B) or Krox-24 (C) IHC. Staining is almost exclusively nuclear, except for weak staining of fibers in the Krox-24 sections, giving these sections a darker appearance. After injection of vehicle and NRTN, a few scattered c-Fos-ir and c-Jun-ir cells are visible throughout the dorsal horn, while Krox-24 is mainly expressed in lamina III and IV (C1). After injection of GDNF, BDNF and NMDA, the increase in IEG expression compared to vehicle and NRTN is most pronounced in the superficial dorsal horn, while a smaller increase in c-Fos and Krox-24 can be observed in the deeper layers of the dorsal horn. C-Jun expression in the deep dorsal horn was not clearly increased after GDNF and BDNF injection. Lamina II-inner was almost devoid of IEG-ir cells after injection of GDNF and BDNF (A3, B3, C3).


c-Fos
c-Jun
Krox-24
vehicle
NTN
GDNF
BDNF
NMDA
A1
B1
C1
A2
B2
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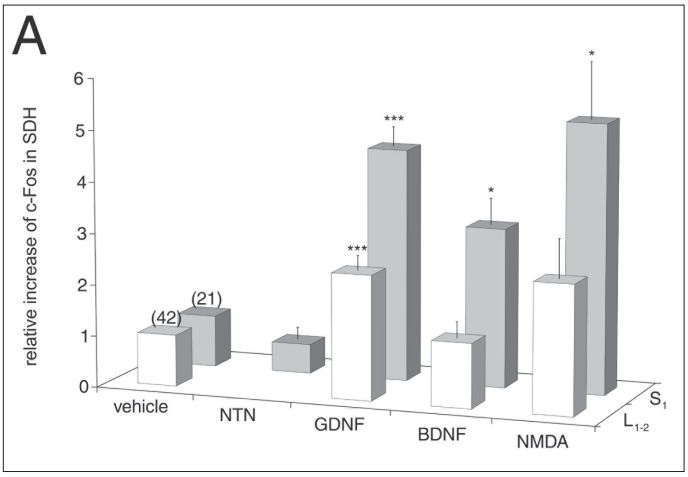
C3
A4
B4
C4
A5
B5
C5
120μm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vehicle
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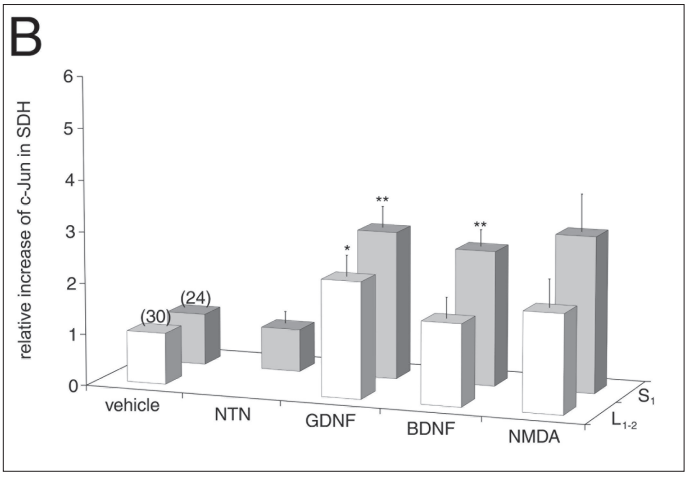
L1-2


B
relative increase of c-Jun in SDH
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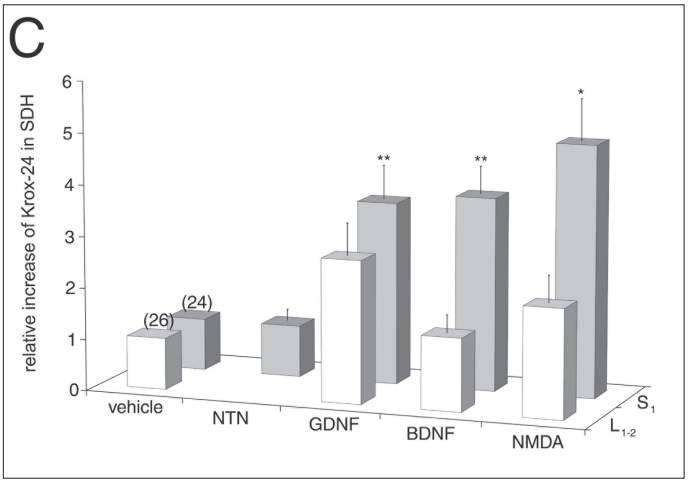
C
relative increase of Krox-24 in SDH
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*
vehicle
NTN
GDNF
BDNF
NMDA
S1
L1-2

Fig. 3. (previous page) Quantification of IEG expression in the superficial dorsal horn at S1 and at L1/L2 after intrathecal injections. Values are means ± SEM of relative increase of c-Fos-ir (A), c-Jun-ir (B) and Krox-24 (C)-ir cells in the superficial dorsal horn, i.e. lamina I and II, after NRTN, GDNF, BDNF and NMDA injection. The values between brackets represent the means of the absolute numbers of cells after vehicle injection. Significance levels: *p≤0.05; **p≤0.01; ***p≤0.001, versus *vehicle*, unpaired t-test.

Relative increase of c-Fos in the deep dorsal horn at S1 and at L1/L2 following intrathecal injections

	vehicle		NTN		GDNF		BDNF		NMDA	
	S1	L1/L2	S1	L1/L2	S1	L1/L2	S1	L1/L2	S1	L1/L2
lamina III-IV	1(7)	1(18)	0.6 ± 0.3	nd	5.4 ± 2.9*	1.5 ± 0.4*	2.0 ± 0.4	1.4 ± 0.4	6.3 ± 1.9*	2.0 ± 0.6
lamina V	1(9)	1(15)	0.9 ± 0.6	nd	2.7 ± 1	1.1 ± 0.1	1.6 ± 0.4	1.1 ± 0.5	2.2 ± 0.6	1.5 ± 0.4

Relative increase of c-Jun in the deep dorsal horn at S1 and at L1/L2 following intrathecal injections

lamina III-IV	1(13)	1(17)	1.2 ± 0.5	nd	1.4 ± 0.5	1.3 ± 0.5	0.7 ± 0.3	1.4 ± 0.2	1.8 ± 0.4	1.1 ± 0.4
lamina V	1(14)	1(13)	0.8 ± 0.2	nd	0.9 ± 0.4	1.2 ± 0.3	0.5 ± 0.2	1.8 ± 0.4	0.5 ± 0.1	1.3 ± 0.7

Relative increase of Krox-24 in the deep dorsal horn at S1 and at L1/L2 following intrathecal injections

lamina III-IV	1(33)	1(68)	1.1 ± 0.2	nd	1.9 ± 0.3*	1.3 ± 0.2	1.6 ± 0.1	1.3 ± 0.3	2.9 ± 0.8*	1.0 ± 0.3
lamina V	1(22)	1(24)	1.2 ± 0.5	nd	1.9 ± 0.3*	1.1 ± 0.1	1.7 ± 0.3	0.8 ± 0.1	2.2 ± 0.5	1.4 ± 0.5

Table. Quantification of IEG expression in the deep dorsal horn at S1 and at L1/L2 after intrathecal injections.

Values are means ± SEM of relative increase of c-Fos-ir, c-Jun-ir and Krox-24-ir cells in the deep dorsal horn, i.e. lamina III and IV and lamina V after NRTN, GDNF, BDNF and NMDA injection. The values between brackets represent the means of the absolute numbers of cells after vehicle injection. Significance levels: *p≤0.05; **p≤0.01; ***p≤0.001, versus *vehicle*, Mann-Whitney's rank-sum test.

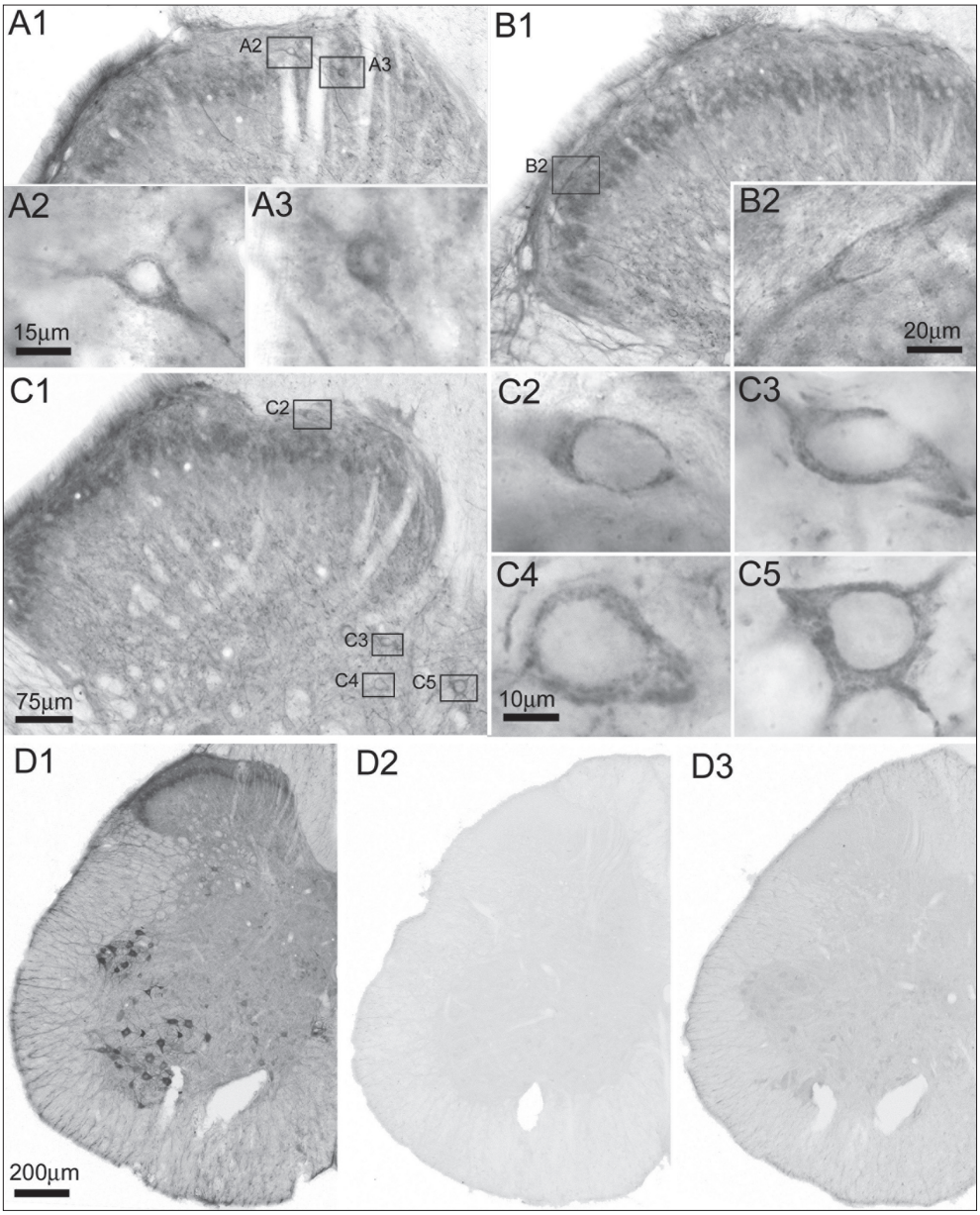

Fig. 4. Light micrographs, showing the dorsal horn of S1 sections from naïve rats processed for RET IHC. RET-immunoreactivity is localized in motoneurons (D1) and presumed terminals in lamina II-inner (A1, B1, C1, D1), but also in the cytoplasm and primary dendrites of neurons in the superficial (A1-3, B1-2, C1-2) and deep (C1, C3-5) dorsal horn. Some cells in lamina I appeared as fusiform neurons, possibly represening nociceptive specific neurons (B2, C2). Control sections in which the primary antibody was omitted (D2) or preincubted with the synthetic protein the antibody was raised aganst (D3) showed virtually no immunohistochemical staining.

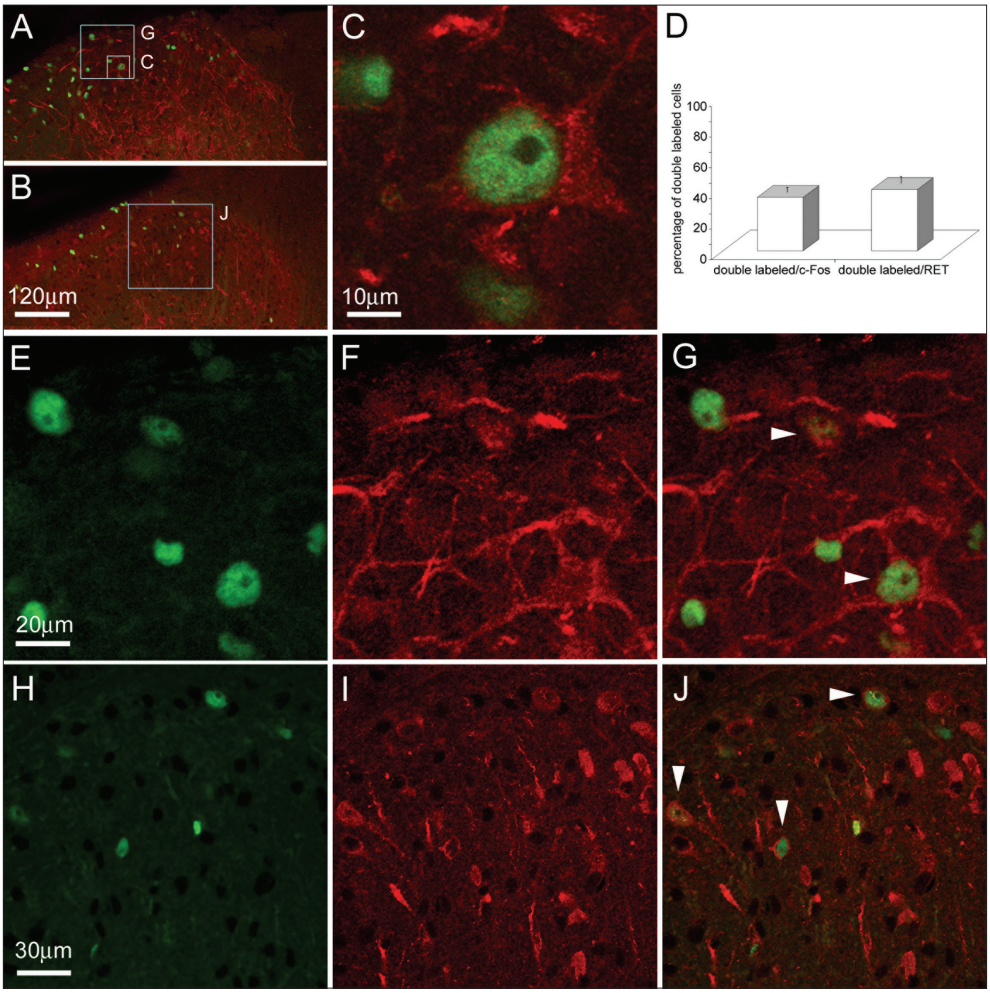

Fig. 5. Confocal IF images of the superficial dorsal horn of S1-sections from rats injected intrathecally with 10 μg GDNF (A, B). c-Fos is labeled with FITC (green; E, H) and RET is labeled with Cy3 (red; F, I). Superimposed images (C, G, and J) showing double-labeled neurons (arrowheads). The percentage of double-labeled neurons as a proportion of total number of c-Fos-ir cells and RET-ir neurons in the lateral half of lamina I and II, where the large majority of c-Fos-labeled cells was present, is shown in D. Thickness of optical sections: 4.6 μm (A-G) and 1 μm (H-J).

Chapter 5

Distribution of RET Immunoreactivity in the Rodent Spinal Cord and Changes after Nerve Injury

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ABSTRACT

RET (REarranged during Transfection) is a transmembrane tyrosine kinase signaling receptor for members of the glial cell line-derived neurotrophic factor (GDNF) family of ligands. We used RET immunohistochemistry (IHC), double labeling immunofluorescence (IF) and *in situ* hybridization (ISH) in adult naïve and nerve injured rats to study the distribution of RET in the spinal cord.

In the dorsal horn, strong RET-immunoreactive (-ir) fibers were abundant in lamina II-inner (II_i) and clusters of varicose fibers were found in the deeper layers, although this labeling was preferentially observed after an antigen unmasking procedure. Following dorsal rhizotomy, the fibers in lamina II_i and the varicose fibers completely disappeared from the dorsal horn, indicating that they were all primary afferents. Following peripheral axotomy, RET-ir in primary afferents decreased in lamina II_i and appeared to slightly increase in lamina III and IV. RET-ir was also observed in neurons and dendrites throughout the dorsal horn. Some RET-ir neurons in lamina I had the morphological appearance of nociceptive projection neurons, which was confirmed by the finding that 54% of RET-ir neurons in lamina I colocalized with neurokinin-1.

In the ventral horn, RET-immunoreactivity (-ir) was strongly expressed by motoneurons, with the strongest staining in small, presumably gamma-motoneurons, since they did not express cholinergic boutons and m2 muscarinic acetylcholine receptor-ir on their plasma membrane, in contrast to alpha-motoneurons. Increased RET expression following peripheral axotomy was most pronounced in alpha-motoneurons.

The expression and regulation pattern of RET in the spinal cord is in line with its involvement in regenerative processes following nerve injury. The presence of RET in dorsal horn neurons, including nociceptive projection neurons, suggests that RET also has a role in signal transduction at the spinal level. This role may include mediating the effects of GDNF released from nociceptive afferent fibers.

Indexing Terms: axotomy; dorsal horn; Glial cell-line derived neurotrophic factor; immunohistochemistry; *in situ* hybridization histochemistry; motoneurons; neurokinin-1; pain; rhizotomy; rat

RET (REarranged during Transfection) is a proto-oncogene, originally identified by DNA rearrangement (Takahashi et al., 1985), encoding a tyrosine kinase receptor protein. After its initial discovery RET was found to play many, often diverse, roles. Mutations leading to a constitutively active form of RET are involved in producing human papillary thyroid carcinoma (Grieco et al., 1990) and multiple endocrine neoplasia (Hofstra et al., 1994; Mulligan et al., 1993), while inactive RET leads to various developmental disturbances, including Hirschsprung's disease and renal dysgenesis (Schuchardt et al., 1994). In 1996 it was found that Glial cell line-Derived Neurotrophic Factor (GDNF) was the endogenous ligand for the RET tyrosine kinase receptor (Durbec et al., 1996; Trupp et al., 1996), and that a Glycosyl PhosphatidylInositol (GPI)-linked ligand binding subunit, now known as GDNF Family Receptor-α1 (GFRα1), was needed for activating RET tyrosine kinase by GDNF (Jing et al., 1996; Treanor et al., 1996). More recent findings have shown that other members of the GDNF family of neurotrophic factors, also known as GDNF-family ligands (GFLs), i.e. Neurturin (NRTN) (Kotzbauer et al., 1996), Artemin (ARTN) (Milbrandt et al., 1998) and Persephin (PSPN) (Baloh et al., 1998), also use RET as their signal transducing element, with GFRα2, GFRα3 and GFRα4 respectively as their preferential co-receptor (Airaksinen and Saarma, 2002).

Lipid rafts and localization of RET

The last decade has shown accumulating evidence that parts of the neuronal cell surface are organized into lipid based microdomains, also known as lipid rafts: islands of highly ordered sphingolipids and cholesterol on the outer leaflet of the plasma membrane (Munro, 2003; Simons and Toomre, 2000; Tsui-Pierchala et al., 2002). RET in neurons can be localized inside or outside lipid rafts. When a GFL binds to its cognate GFRα, which is preferentially localized inside rafts, the complex will recruit RET to the raft, where the GFL-GFRα-RET complex is stabilized. These events, also known as *in cis* signaling, preferentially occur when RET and GFRα are produced by the same cell (Paratcha and Ibanez, 2002; Tansey et al., 2000; Yu et al., 1998). When GFRα originates from a source outside the cell arriving through the intracellular space, it will initially form a GFL-GFRα complex and bind RET in the membrane outside rafts (*in trans* signaling) and the complex may subsequently translocate to a raft (Paratcha and Ibanez, 2002; Paratcha et al., 2001). The distinction between *in cis* and *in trans* signaling is important, since signaling properties of RET in the lipid raft environment differ from those when RET is located outside lipid rafts (Paratcha and Ibanez, 2002).

The GFLs, their GFRα co-receptors and RET, have all been identified in the developing and adult peripheral and central nervous system, mostly by using ISH (Glazner et al., 1998; Golden et al., 1998; Golden et al., 1999; Nosrat et al., 1997). RET expressing structures include the sympathetic and parasympathetic ganglia (Enomoto et al., 2000; Nishino et al., 1999; Rossi et al., 2000), midbrain dopaminergic neurons (Trupp et al., 1997), dorsal root ganglion (DRG)

and trigeminal ganglion neurons (Bennett et al., 2000; Bennett et al., 1998; Golden et al., 1999; Holstege et al., 1998; Molliver et al., 1997; Naveilhan et al., 1998; Orozco et al., 2001; Widenfalk et al., 1998) and the spinal cord, most notably motoneurons (see later). RET is generally expressed in two isoforms, RET9 and RET51 (Coulpier et al., 2002; de Graaff et al., 2001; Wong et al., 2005). In the present study, as in most studies, probes were used that did not distinguish between the two isoforms, and recognized both.

RET in DRG and spinal cord

In the DRG RET is expressed by both large and small diameter DRG neurons (Bennett et al., 1998; Molliver et al., 1997). Small diameter DRG neurons only start to express RET postnatally, when these ganglion cells switch from expressing the Nerve Growth Factor (NGF) receptor TrkA to expressing RET (Molliver et al., 1997). These small size DRG neurons are further characterized by co localization of the plant lectin IB4 and one or more of the GFRαs (Bennett et al., 1998; Orozco et al., 2001). Although the exact functional role of the various RET expressing DRG neurons is not clear, large diameter RET-expressing DRG neurons have been associated with mechanosensitivity in the skin (Molliver et al., 1997; Snider and McMahon, 1998), while small diameter RET- and IB4-expressing DRG neurons have unmyelinated fibers and are involved in nociception (Boucher et al., 2000; Gardell et al., 2003; Stucky et al., 2002).

Several studies have identified RET protein or mRNA in the adult spinal cord in two specific neuronal structures: in motoneurons (Glazner et al., 1998; Golden et al., 1998; Golden et al., 1999; Josephson et al., 2001; Leitner et al., 1999; Nosrat et al., 1997; Widenfalk et al., 2001) and in fibers terminating in the inner part of lamina II (Bennett et al., 1998; Molliver et al., 1997), originating from DRG neurons as described above. In motoneurons, RET serves as part of the receptor complex involved in retrograde signaling as part of the survival, maintenance and regeneration machinery of the neuron (Boyd and Gordon, 2003; Henderson et al., 1994; Leitner et al., 1999; Li et al., 1995; Oppenheim et al., 1995; Vejsada et al., 1998; Yan et al., 1995; Yuan et al., 2000; Zhao et al., 2004), which becomes activated by GFLs originating from muscle and Schwann cells. Early studies, which did not mention gamma motoneurons, suggested that RET is specifically expressed in alpha motoneurons, (Glazner et al., 1998; Nosrat et al., 1997), although this has never been confirmed. Damage of motor axons leads to increased RET expression in motoneurons (Burazin and Gundlach, 1998; Hammarberg et al., 2000; Homma et al., 2003; Naveilhan et al., 1997; Tsujino et al., 1999), following a short-lived increase in GDNF expression in damaged nerve Schwann cells (Hoke et al., 2002; Naveilhan et al., 1997) and a delayed increase in skeletal muscle (Naveilhan et al., 1997). In these circumstances, GDNF appears to be involved in promoting terminal axon branching and synapse formation. During aging there is also increased RET expression (Bergman et al., 1999), possibly compensatory to decreased release of trophic factors from muscle.

Aim of the study

In most studies cited so far, the role of RET is interpreted as signaling the effects of GFLs, acting as retrograde neurotrophic factors. However, we have found evidence that suggests that in the spinal cord GDNF may act as a neuromodulator, which is released from primary afferent fibers and is involved in sensory processing in the dorsal horn (Holstege et al., 1998; Jongen et al., 2005; Jongen et al., 1999).

GDNF is present in dense cored vesicles in primary afferent fibers terminating in laminae I and II-outer of the spinal dorsal horn and in the spinal trigeminal nucleus (Del Fiacco et al., 2002; Holstege et al., 1998; Ohta et al., 2001; Quartu et al., 1999). Furthermore, exogenously applicated GDNF can rapidly induce c-Fos in dorsal horn neurons (Jongen et al., 2005). If GDNF, released from primary afferent fibers, is to activate spinal neurons, it would be expected that RET is expressed in the postsynaptic targets of the GDNF containing primary afferent fibers. However, so far the presence of RET protein in dorsal horn neurons has not been described in detail and data on the presence of RET-mRNA in dorsal neurons are conflicting (Glazner et al., 1998; Golden et al., 1998). Therefore, we have initiated the present study to examine in detail the anatomical and functional expression of RET protein in the adult rat spinal cord, with a focus on the dorsal horn, using immunohistochemistry with an antibody recognizing both isoforms of RET and *in situ* hybridization (ISH). In addition we analyzed the expression of RET in other areas of the spinal cord, including motoneurons. Finally, we studied changes in the expression of RET protein in the spinal cord following peripheral axotomy and dorsal rhizotomy.

MATERIALS AND METHODS

Animals and surgery

Experiments were performed on a total number of 22 adult male Wistar rats (Harlan Netherlands B.V., Horst, NL), weighing 250-300 g. Additionally, mice with a targeted mutation in the RET gene (*RET*$^{k-}$; see (Schuchardt et al., 1994)) were used along with their wild type littermates (MRC, London, UK). Experiments were performed in accordance with the 'Principles of Laboratory Animal Care' (NIH publication no. 86-23). Local Animal Ethics Committees had approved all experiments.

Peripheral axotomies were performed on six rats under general anesthesia with 2% halothane in O_2/N_2O (30/70%). The left sciatic nerve was exposed, dissected free and locally anaesthetized with xylocaine 1%/adrenaline 1:100,000 (Astra Pharmaceutica BV, Zoetermeer, NL). Subsequently, the nerve was transected and 5 mm was removed at the midthigh level to

prevent regeneration of the proximal stump into the distal stump. After 9 days survival the rats were euthanized, dissected and immunohistochemistry was performed as described below.

Dorsal root transections were performed on four rats under general anesthesia. At the L2-L3 vertebrae a laminectomy was performed and the L2-S2 dorsal roots, which pass at this level, were anesthetized with xylocaine/adrenaline and then transected by removing 1-2 mm of nerve. After 6 days survival the rats were euthanized, dissected and immunohistochemistry was performed as described below.

Immunohistochemistry and immunofluorescence

For immunohistochemistry (IHC) and immunofluorescence (IF), rats were deeply anesthetized with intraperitoneal (i.p.) sodium pentobarbital and perfused transcardially with 250 ml phosphate buffer (PB; 0.05M, pH 7.3), containing 0.8% NaCl, 0.8% sucrose and 0.4% D-glucose, followed by 600 ml of 0.05M PB containing 4% paraformaldehyde (PFA) and 4% of the same fixative also containing 15% sucrose. The lumbosacral spinal cord was removed and post fixed in 4% PFA/15% sucrose for 2 hours (h) at room temperature. Some sections were post fixed in the same fixative for an additional 2 hours at 4^{0}C the same day. 40μm Frozen sections were cut on a sliding microtome, collected in 0.1 M PB and processed as free floating sections. Sections from animals with the longer post fixation period were heated at 80^{0}C in a 0.025M sodium citrate buffer solution (pH 8.75), in order to unmask immunoreactivity (see also (Jiao et al., 1999).

A *RET*$^{k-/+}$ pregnant female bearing *RET*$^{k-}$ mice and their wild type littermates, was transcardially perfused at day 17.5 of pregnancy, in the same way as described for adult rats. Embryos were dissected, genotyped and spines were removed and post fixed in 4% paraformaldehyde for two hours at room temperature. Whole spines from a homozygous *RET*$^{k-}$ mouse embryo and from a wild type littermate were embedded in gelatin blocks and 40 μm frozen sections were cut on a sliding microtome. All sections were pretreated by heating them in sodium citrate buffer.

For *single-labeling RET immunohistochemistry* on rats and mice, all sections were preincubated with 10% normal horse serum (NHS) in 0.05M tris-buffered saline (TBS) containing 0.5% Triton X-100, followed by incubation (40 hours at 4^{0}C) in TBS (pH 7.6) containing 1% NGS, 0.1% Triton X-100 and goat anti-mouse RET (R&Dsystems, Minneapolis, MN; see also (Jongen et al., 2005), diluted 1:100 (heated sections) or 1:50 (unheated sections). Sections were then rinsed (4x) in TBS, incubated for 90 minutes in biotinylated donkey anti-goat (Vector, Burlingame, CA) diluted 1:200 in TBS also containing 2% NHS and 0.4% Triton X-100, rinsed again in TBS (4x), processed with the ABC method (Vector Elite) and reacted with 0.025% diaminobenzidine, containing 0.005% hydrogen peroxide. To control for the specificity of the primary antibody and the immunohistochemical procedure the following control experiments were performed: omission

of the primary antibody, preincubation with a 10x (by weight) excess of the synthetic protein the antibody was raised against (R&D systems) and staining of spinal cord/DRG sections of a *RET*$^{k-}$ mouse embryo (see also (Jongen et al., 2005).

For *double- and triple-labeling experiments*, the same protocol as for RET single-labeling immunohistochemistry was used, without heating. Other primary antibodies included: Anti-microtubule-associated protein 2 (MAP2) polyclonal antibody raised in rabbit (Chemicon, Temecula, CA; see also (Niu et al., 2004) used at a dilution of 1:100, anti-neurokinin-1 (NK-1) polyclonal antibody raised in rabbit (kindly provided by Dr. P.W. Mantyh, Neurosystems Center, University of Minnesota, Minneapolis, MN; see also (Mantyh et al., 1995; Vigna et al., 1994) used at 1:5000, rat m2 muscarinic acetylcholine receptor monoclonal antibody (Chemicon; see also (Levey et al., 1995) at 1:100 and mouse choline acetyltransferase (ChAT) monoclonal antibody (mAb 5, kindly provided by Dr. C. Cozzari, Institute for Cell Biology, CNR, Rome, Italy; see also (Jaarsma et al., 1996) at 1:100. Secondary antibodies included Cy3-conjugated donkey anti-goat, FITC-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-rat, FITC-conjugated donkey anti-mouse and Cy5-conjugated donkey anti-goat (Jackson ImmunoResearch, West Grove, PA), all used at a dilution of 1:200, for 1.5 h at 20^{0}C. Vectashield (Vector Laboratories Inc., Burlingame, CA) was used as a mounting medium.

In situ hybridization

For *RET-ISH* four adult Wistar rats received an overdose of sodium pentobarbital and were transcardially perfused with 250 ml sterile saline, followed by 750 ml 4% PFA in 0.12 M PB (pH 7.4). The lumbar segment was removed and post-fixed overnight at 4^{0}C in RNAse-free fixative, i.e. 4% PFA/30% sucrose in diethylpyrocarbonate (DEPC)-treated PB. 40μm Frozen sections were cut on a sliding microtome, collected in RNAse free PB and processed as free floating sections. Sections went through the following steps before prehybridization: 5 minutes in 0.2% glycine (Sigma-Aldrich, St. Louis, MO); 10 minutes in 0.1 M triethanolamine (pH 8) (Merck, Whitehouse Station, NJ) and 0.0025% acetic anhydride (Sigma), all in DEPC-treated and autoclaved phosphate buffered saline (PBS). After washes in 4x standard saline citrate (SSC), sections were transferred to an aqueous hybridization solution containing 50% formamide, 5x SSC, 2% blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany), 0.05% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sigma), 1 μg/ml yeast tRNA, 5 mM ethylenediaminetetraacetic acid (EDTA), 50 μg/ml heparin and 1x Denhardt's solution (Sigma), for 1 hour at 65 ^{0}C. The riboprobe for RET mRNA was directed against the extracellular domain of mouse RET and encompassed the first 1.8 kb SacII-SalI fragment (kindly provided by Dr. V. Pachnis, Division of Molecular Neurobiology, MRC, London, UK). The RET plasmid was linearized with BamHI and transcribed with T3 RNA polymerase in the presence of digoxigenin-11-UTP (Roche). Sections were incubated in hybridization solution for 16 hours at 65^{0}C. Subsequently

sections were rinsed in 2x SSC, treated three times with a solution of 50% formamide and SSC, at 65 ^{0}C and rinsed in PBS. Sections were then processed for detection of digoxigenin-incorporated RET riboprobes with a sheep polyclonal antibody conjugated to alkaline phosphatase (1:4000; Roche) in a 2% BSA, 0.5% Triton, 0.05 M PBS solution for 48 h at 4^{0}C. Alkaline phosphatase was reacted with nitroblue tetrazolium and 5-bromo-4-chloro-3-indonyl-phosphate (Roche) for 9 hours in the dark at room temperature. After rinsing, sections were mounted, air-dried overnight, dehydrated with absolute ethanol (VWR, West Chester, PA) and coverslipped with Permount as a mounting medium (Fisher, Hampton, NH). Control sections, hybridized with sense probes, were also analyzed but did not show any specific labeling.

Image analysis and quantification of immunohistochemical data

To analyze the degree of *RET and NK-1 co localization*, we used double labeling immunofluorescence combined with confocal microscopy on L5 sections that were 200 μm apart, from 5 rats. Lamina I was systematically scanned for RET-ir neurons, NK-1-ir neurons and double labeled neurons, using a Zeiss LSM 510 confocal laser scanning microscope and a 20x objective. Thickness of optical sections was 4.6 μm. A total number of 201 immunoreactive cells were counted. The percentages of double labeled neurons as a proportion of total number of RET-ir and NK-1-ir neurons were expressed as means ± SEM.

For *quantification of regulation of RET-ir* in lamina II_i and III following peripheral axotomy, the first four L5 sections (each 200 μm apart) encountered on slides from 5 axotomized animals were photographed with a Leica DC 300 digital camera on a Leica DMRB light microscope. The resulting images ($n=20$) were further analyzed using the public domain ImageJ (v1.33) software, a Java application based on NIH image (http://rsb.info.nih.gov/nih-image/about.html). A square selection of 64x64 pixels containing part of the medial half of lamina II_i and a similar selection ventral to the former, containing a part of lamina III, was selected on both sides. Processing and image analysis was performed as described previously (Wu et al., 1997), with the exception of transition to and from the frequency domain. This method creates an image that appears black against a white background, which is an accurate representation of RET-ir in the selected area. The labeled area was expressed as the number of black pixels. Mean values of 5 animals were calculated for control and axotomy sides and were compared using a paired *t*-test.

Quantification of RET-ir in motoneurons was performed on the first L5 section encountered on the slide (each 200 μm apart) from the same 5 axotomized animals as above. The resulting images ($n=5$) were also preprocessed according to (Wu et al., 1997) and immunoreactive profiles in the ventrolateral spinal gray matter containing a nucleus, i.e. motoneurons, were selected using the freehand selection tool. Feret diameter of motoneuronal somata (in μm) and mean gray value of the cytoplasm of motoneuronal somata (ranging form 0-255) were measured with ImageJ.

Mean values of 5 animals were calculated for control and axotomy sides. Additionally, mean values from three naïve rats were determined. Thus, a total number of 318 RET-ir motoneurons were analyzed. For quantification motoneurons were grouped in 5 μm Feret diameter intervals and proportions of RET-ir neurons with intensities < 110 (i.e. darkly stained) were calculated for each group. Motoneurons with diameters of < 25 and ≥ 60 μm were discarded from analysis, since too few data were available to make estimates of proportions reliable. *t*-Test statistics were used to compare density of RET staining in control sides of axotomized rats with that in naïve rats, to compare proportions of darkly stained RET neurons in small size motoneurons with a diameter of <40μm with that in medium-large size (≥40 μm diameter) motoneurons and to compare the axotomy side with the control side of axotomized animals.

Statistical analysis was performed with Stata v8.0 software (www.stata.com). Figures were composed with Adobe Photoshop v7.0 software. Adjustments were made only to brightness and contrast, unless otherwise indicated in the figure legends. Evenness of illumination of light microscopical sections was provided by background subtraction using the TWAIN software that came with the Leica DC 300 digital camera.

RESULTS

RET general labeling pattern and epitope unmasking

Light microscopical examination of the sections processed for RET-IHC showed that at low-magnification, RET-ir was most prominent in lamina II_i and in mooneurons (Fig. 1A). Many other neurons were also RET-ir, but with a much lower intensity than motoneurons. These weakly RET-ir neurons were found in all laminae, except for lamina II, which contained only a few labeled neurons. The many fiber-like structures that were labeled throughout the spinal cord were mainly dendrites, since virtually all of these structures also expressed the dendritic marker MAP2 (see later). However, the fibers in lamina II_i were primary afferent fibers terminating in this area, since these fibers disappeared after dorsal rhizotomy (see later). This general labeling pattern was seen along the entire length of the spinal cord. The prominent RET labeling in primary afferent fibers in lamina II_i was preferentially observed after pretreatment of the sections at 80^0C in sodium citrate buffer, the so-called unmasking procedure. When this treatment was omitted, only labeling of neuronal somata and dendrites was observed (Fig. 1B), demonstrating that the unmasking procedure only affects RET protein associated with axonal and terminal structures.

In the spinal cord of an E 17.5 wild type mouse strong RET-ir was observed in DRG neurons, motoneurons and primary afferent terminals (Fig. 1D), especially after the unmasking procedure. Without this treatment, labeling of primary afferent terminals was nearly absent (Fig. 1E), exactly

as observed in the adult rat, while labeling of motoneurons and DRG neurons was also somewhat weaker. RET-ir primary afferent fibers at this age, when the dorsal horn and its connections are still in development, were present throughout lamina I-IV of the dorsal horn, as described previously (Molliver et al., 1997). A limited number of RET-ir neurons were also identified in the dorsal horn, although the multitude of afferent fibers made it difficult to ascertain their presence. In a mouse with a targeted mutation in the gene encoding the RET protein (Schuchardt et al., 1994), which lack normal RET mRNA, no labeling in any of the aforementioned structures was observed after pretreatment for epitope unmasking (Fig. 1C), confirming the specificity of the antibody. However, there was some labeling of structures resembling radial glia in the embryonic mice lacking RET protein. Although this labeling was relatively weak, it prompted us to consider labeling in glial structures as non specific.

We also used non-radioactive ISH on free floating sections (Key et al., 2001) to study the presence of RET mRNA in the spinal cord (Fig. 2). In the ventral horn, strong RET-mRNA labeling was present in motoneurons (Fig. 2C), while in the dorsal horn, several weakly, but still distinct, RET mRNA expressing neurons were present in the superficial (Fig. 2A and 2B) and deep (not shown) layers. Generally, the distribution of labeled neurons corresponded very well with the general neuronal distribution, obtained with RET-IHC (compare Figs. 1, 2 and 4).

The general distribution and relative intensity of RET-ir in the spinal cord is summarized in the table. Specific spinal nuclei showing RET-ir neurons (Fig. 3) were the lateral spinal nucleus, the dorsal nucleus (nucleus dorsalis of Clarke; thoraco-lumbar spinal cord), the intermediolateral nucleus (thoraco-lumbar spinal cord), the sacral parasympathetic nucleus and the nucleus dorsomedialis and dorsolateralis (homologues to Onuf's nucleus in man; L6 segment) (see also (Holstege et al., 1996). Tanycytes (see also (Honda et al., 1999) around the central canal also showed RET-ir.

RET expression in dorsal horn neurons

Several strongly RET-ir neurons and a significant number of more weakly labeled neurons were observed scattered throughout the entire dorsal horn (Fig. 4A). Typically, each section contained several RET-ir neurons in lamina I, some of which had the characteristic appearance of nociceptive projection neurons (Fig. 4B) (see also (Grudt and Perl, 2002; Han et al., 1998). RET-ir neurons were relatively abundant in lamina V and sparse in lamina II. Lamina III, IV and V contained, in addition to somatic RET labeling, many RET-ir fiber-like structures that were not affected by dorsal rhizotomy. These structures represented dendrites, as double-labeling IF combined with confocal microscopy showed that virtually all of them were immunoreactive for RET as well as for the dendritic marker MAP2 (Fig. 5). Since MAP2 is selectively expressed in neuronal somata and dendrites and not in glia, the extensive co localization of RET and MAP2 immunoreactivity further demonstrates that labeled structures in the dorsal horn are neuronal rather than glial.

Using double-labeling confocal IF, it was determined whether RET-ir neurons in lamina I were also NK-1-ir. A quantitative analysis of these neurons showed that 54 ± 4% (mean ± SEM) of the RET labeled neurons were also labeled for NK-1 and that 65 ± 4% (mean ± SEM) of the NK-1 labeled neurons were also labeled for RET (Fig. 6).

RET expression in primary afferent fibers

A dense band of RET-ir fibers was observed in lamina II_i and clusters of varicose fibers were observed in the medial part of lamina IV and V, especially in sections that were pretreated using antigen unmasking. These RET-ir fibers represented primary afferent terminals, since a L2-S2 dorsal rhizotomy resulted in a nearly complete disappearance of RET-ir fibers in lamina II_i of the L4-L6 segments (Fig. 7).

In the dorsal horn of rats that were subjected to an axotomy of the sciatic nerve at thigh level 10 days previously, a 45% decrease ($P \leq 0.05$) of RET labeling density was observed in the medial half of lamina II_i on the axotomized side (Fig. 8A, C), which is the projection area of RET-ir sciatic nerve afferents. A simultaneous increase of RET-ir was observed in the adjacent medial half of lamina III and IV on the axotomized side. However, a density measurement of this increase in RET-ir in the medial half of lamina III just failed to reach statistical significance ($P=0.08$). The apparent increase was not observed when sciatic nerve axotomy was followed by dorsal rhizotomy of L2-S2, suggesting that the apparent increase in lamina III and IV labeling after peripheral axotomy occurred in primary afferent fibers rather than in neuronal somata and dendrites (Fig. 8B, C).

RET expression in motoneurons.

RET-ir was strongly expressed in motoneurons throughout the rat spinal cord (Fig. 1, 9). This was confirmed by RET and ChAT double-labeling IF, since virtually all motoneurons, as identified by their ChAT labeling, were also labeled for RET (Fig. 10A). RET labeling was prominent in the cytoplasm with sparing of the nucleus and extended into the dendrites. Generally, RET-ir was much stronger in small (diameter < 40 μm) than in large-sized (diameter ≥ 40 μm) motoneurons (Fig. 9). The proportion of motoneurons with intensities lower than 110 on a 256 gray level scale (i.e. darkly stained motoneurons) was almost twice as high in the group of small motoneurons, than in the group of large motoneurons (1.9 times; $P \leq 0.01$, group 25-40 μm versus 40-60 μm diameter, paired *t*-test; see also Fig. 11C). Small, strongly RET-ir neurons co expressed m2 receptor-ir weakly or not at all and did not show cholinergic boutons on their plasma membrane (Fig. 10B).

In the ventral horn, 10 days following sciatic nerve axotomy, an almost twofold increase in the proportion of darkly RET-labeled motoneurons was observed on the axotomized side as compared to the control side (Fig. 11; 1.8 fold; $P \leq 0.01$). The increase was almost entirely due to increased labeling in large motoneurons (Fig. 11 A, C). RET labeling intensities of motoneurons on the control sides of axotomized rats did not differ from those in naïve rats nor did motoneuron diameters ($P=0.88$, $P=0.14$ respectively; data not shown).

DISCUSSION

In this study we have used immunohistochemistry to identify the distribution of RET protein in the adult rat spinal cord. The strongest expression was found in small diameter motoneurons, presumably gamma motoneurons, while slightly weaker RET expression was observed in alpha motoneurons. RET-ir in motoneurons was confirmed by ISH. Strong labeling was also observed in primary afferent fibers after an unmasking procedure. Weaker but distinct RET labeling was observed in neurons of nearly all laminae, especially in the dorsal horn. The cellular immunohistochemical expression pattern in the dorsal horn was confirmed by weak, yet distinct, RET mRNA expression.

A potential concern regarding any immunohistochemical study is the specificity of the antibody that is used. In the present study our main data were obtained with a polyclonal antibody produced in goats and raised against the extracellular domain of recombinant mouse RET protein. The antibody has been previously tested (de Graaff et al., 2001) and was found to identify both RET isoforms, in line with the fact that the antibody is directed against the common extracellular domain of RET. RET-ir was absent in present and previous (Jongen et al., 2005) standard negative controls for the antibody and corresponded well with previous findings regarding RET expression in the spinal cord, i.e. localization in fibers in lamina II_i (Molliver et al., 1997) and in motoneurons (Leitner et al., 1999). We also tested the antibody on prenatal mice lacking RET expression. Various neuronal structures, like DRG neurons, motoneurons and primary afferent fibers, that were strongly labeled in wild type mice, were completely devoid of labeling in mice lacking RET expression, thus confirming the specificity of the antibody. Finally, the distribution pattern of RET mRNA expression corresponded well with the immunohistochemical findings. We thus conclude that the immunohistochemical expression pattern that we observed reflects the actual presence of RET protein in the spinal cord.

We demonstrated, both in adult and embryonic tissue, that primary afferent fibers are best visualized following an antigen retrieval technique, which basically consists of pretreating the sections by heating them in sodium citrate at 80 ^{0}C for 30 minutes (Jiao et al., 1999; Montero, 2003). While the labeling of primary afferent terminals in lamina II_i became much more intense

by this procedure, somatic and dendritic labeling was only slightly affected. It seems likely that this differential effect of pretreatment on the antigenicity of RET protein towards the antibody that we used in this study, is related to the different types of cellular compartments containing RET protein: preterminal axonal and terminal membrane localization on the one hand and somatic and dendritic localization on the other. One explanation for the observed effects may be that RET protein in somata and dendrites is always recognized by the antibody when it is localized in the cytoplasm, while RET protein in the cytoplasmatic membrane is either sparsely present or cannot be recognized by the antibody whether the tissue is pretreated or not. In primary afferent terminals RET protein is preferentially visualized after pretreatment, suggesting that in this case pretreatment was effective in unmasking the relevant epitopes of RET protein, in line with a similar finding obtained elsewhere for NMDA and $GABA_A$-receptor epitopes (Fritschy et al., 1998). Whether this means that RET protein in the cytoplasmatic membrane of dorsal horn neurons is differentially localized with respect to lipid rafts than RET in primary afferent terminals cannot be ascertained. However it is tempting to speculate that the different effects of pretreatment on RET protein expression reflect differences between the role of RET in terminals, i.e. retrograde trophic signaling, and RET in dorsal horn neurons, i.e. local modulation of neurotransmission (see later). These different roles of RET are also compatible with observed differences in RET signaling when localized inside or outside lipid rafts.

RET expression in dorsal horn neurons

Although relatively weak, RET protein and RET mRNA are clearly expressed in neurons of the dorsal horn, especially in laminae I and V. Virtually complete colocalization of RET with the neuron specific marker MAP2 confirms that cellular RET is almost exclusively localized in neurons. In this study, as in a previous study (Jongen et al., 2005) we observed RET-ir in fusiform neurons in lamina I, which by morphology and location likely represent nociceptive specific projection neurons (Han et al., 1998). In order to confirm this idea, we applied double labeling IF for RET and NK-1, the receptor for Substance P. NK-1 was used as a marker, since the large majority of the NK-1-ir neurons in lamina I are known to be nociceptive specific projection neurons, which targets include the thalamus, the lateral parabrachial area, the periaqueductal gray the and caudal ventrolateral medulla (Klop et al., 2005; Littlewood et al., 1995; Spike et al., 2003; Yu et al., 1999). The high degree (i.e. over 50%) of RET and NK-1 co localization that we observed thus demonstrates that the majority of RET-ir neurons in lamina I are involved in nociceptive signaling and are able to respond to activation by GFLs like GDNF, since they contain the RET protein necessary for signal transduction. Although we did not perform co localization experiments to characterize neurons in dorsal horn laminae other than lamina I, the relative abundance of RET-ir neurons in lamina V may suggest its presence in wide dynamic range neurons (Craig, 2003; Craig, 2004; Price et al., 2003), which are also involved in processing nociceptive information.

RET expression in primary afferent terminals

This is the first study to provide direct proof that the strong RET-ir fibers in the dorsal horn, most abundantly in lamina II_i, are primary afferents, since an almost complete disappearance of this labeling was observed following dorsal rhizotomy.

Following peripheral axotomy a different reaction was observed. In this case a strong downregulation of RET-ir in fibers in lamina II_i is accompanied by an apparent upregulation of RET-ir fibers in lamina III and IV. The finding that this upregulation was not present when axotomy is followed by a rhizotomy, strongly suggests that the upregulation occurs in a subgroup of primary afferent fibers. We propose that this subgroup are myelinated Aβ fibers, which preferentially terminate in this laminae and belong to large diameter ganglion cells, possibly involved in mechanosensitivity in the skin. Our findings are in agreement with those of (Bennett et al., 2000), who described an increase of RET mRNA expression in large diameter DRG neurons after peripheral axotomy. The downregulation in one and simultaneous upregulation in another subpopulation of primary afferents may also explain why previous studies (Bennett et al., 2000; Hoke et al., 2002; Kashiba et al., 1998) have reported that the overall RET expression in primary afferents is unaffected by peripheral axotomy. A similar situation has been described for neuropeptides and BDNF (Hokfelt et al., 1994; Michael et al., 1999; Neumann et al., 1996; Noguchi et al., 1995). Taken together, our findings show that primary afferents are the only RET expressing fibers terminating in the spinal cord and that this expression is downregulated after peripheral axotomy in small diameter fibers, while an upregulation appeared to occur in large diameter fibers.

RET in motoneurons

Motoneurons show the most intense RET-ir in the spinal cord. However, within the group of motoneurons, small motoneurons were labeled even stronger than large motoneurons. We propose that these small, intensely labeled motoneurons represent gamma motoneurons, in contrast to previous suggestions that alpha motoneurons show the strongest RET expression (Glazner et al., 1998; Nosrat et al., 1997). Early studies have demonstrated a bimodal size-frequency distribution of motoneurons, with gamma motoneurons representing those with a mean diameter smaller than 35-40 μm (Bryan et al., 1972; Limwongse and DeSantis, 1980; Peyronnard et al., 1986; Strick et al., 1976). We did not observe such a bimodal size-frequency distribution of RET-ir neurons in the ventrolateral gray matter, in agreement with other studies using various immunohistochemical markers (Ramer et al., 2003; Welton et al., 1999). Nevertheless, we did find a clear distinction, based on RET labeling intensity, between neurons with Feret diameters <40 μm, presumably gamma motoneurons, and those with diameters ≥ 40 μm. A similar observation has also been made with respect to ChAT labeling of motoneurons (Barber et al., 1984). Additionally, small strongly RET-ir neurons rarely expressed cholinergic boutons and m2 receptor-ir on their plasma

membrane, also supporting a gamma motoneuronal phenotype (Limwongse and DeSantis, 1980; Welton et al., 1999). These findings may indicate that gamma motoneurons produce larger amounts of RET protein than alpha motoneurons, although the functional significance of this phenomenon remains obscure.

The upregulation of RET-ir in motoneurons that we observed following sciatic nerve transection confirms the finding in numerous other studies, which show an increase in RET mRNA after axotomy, e.g. (Burazin and Gundlach, 1998; Hammarberg et al., 2000; Naveilhan et al., 1997). Upregulation of RET in axotomized motoneurons is generally considered to reflect a compensation for the limited availability of GFLs as a result of the axotomy.

Functional implications: The role of RET in the spinal cord

Based on our previous studies (see introduction) we have argued that GDNF is present in the spinal cord in primary afferent nociceptive fibers from which is it released as a neuromodulator to act on dorsal horn neurons in lamina I and II. This hypothesis is now substantiated by our finding that RET is expressed in NK-1 expressing nociceptive specific projection neurons, which is in line with our previous finding that intrathecal GDNF injection induced c-Fos in neurons that coexpressed RET (Jongen et al., 2005). The expression of GFRα1 in dorsal horn neurons (Glazner et al., 1998; Matsuo et al., 2000; Widenfalk et al., 2001) completes the idea of GDNF as a neuromodulator in spinal nociceptive transmission. The presence of GFRα2 in dorsal horn neurons (Widenfalk et al., 2001) would suggest that NRTN, the preferred ligand for GFR□2, may also play a role in signaling through RET in the spinal cord. However, so far NRTN has not been identified in the dorsal horn or in dorsal root ganglion cells and, after intrathecal injection, does not induce immediate early gene expression, in contrast to GDNF (Jongen et al., 2005). GFR□3 (Widenfalk et al., 1998) and GFRα4 have not been described in the spinal dorsal horn, nor have their ligands ARTN and PSPN. Thus, NRTN, ARTN and PSPN are unlikely to play a role in spinal nociceptive transmission.

Although the nature of an effect of GDNF on dorsal horn neurons is still unclear, spinal intrathecal application of a GDNF-function blocking antibody attenuated the hyperalgesia induced by complete Freund's adjuvans injection in rats (Fang et al., 2003) and a more recent observation has shown acute effects of intrathecally injected GDNF on thermal nociceptive thresholds in mice (Beidas et al., 2005). In the brain GDNF has been shown to directly modulate ion channels (Wang et al., 2003; Yang et al., 2001), although the role of RET or any other receptor in this process is still unclear. Modulation of ion channels may explain the direct behavioral effects of GDNF described above. Similar effects, on NMDA receptors (Kerr et al., 1999) in the spinal cord and on sodium channels in the brain (Blum et al., 2002), have also been described for BDNF. In summary, we suggest that GDNF, like BDNF, may exert a neuromodulatory role in spinal nociceptive transmission, through a direct effect on RET expressing dorsal horn neurons.

In contrast to the possible effect of GDNF released from primary afferent fibers on spinal dorsal horn neurons discussed above, it has been suggested that GDNF acts on RET and GFRα1 expressing primary afferents. However the expression areas of GDNF-ir and RET-ir primary afferents in the spinal cord are hardly overlapping (Bennett et al., 1998; Holstege et al., 1998; Molliver et al., 1997). In pathological situations on the other hand, like inflammation or nerve injury, GDNF and other GFLs might be up regulated in spinal cord glia, as has been shown for BDNF (Coull et al., 2005), exerting trophic effects on DRG afferents. Exogenous application of GDNF (Bennett et al., 1998; Boucher et al., 2000) and ARTN (Gardell et al., 2003) have actually been shown to exert such effects on DRG neurons.

In the ventral horn, RET is involved in the survival of motoneurons during development and in regeneration following damage in the adult. GDNF and NRTN act as trophic factors released by target tissue, i.e. muscle (Trupp et al., 1997; Trupp et al., 1995; Widenfalk et al., 1997) and supporting cells, i.e. Schwann cells (Hoke et al., 2002) and possibly glial cells in the ventral horn (Zhao et al., 2004). These effects are mediated by RET and GFRα1 or GFRα2, which are all present in motoneurons (see introduction), although the exact role of these receptors in this trophic process is largely unknown. Nevertheless, GDNF has been extensively used as a therapeutic in animals models of motoneuron disease (Bohn, 2004) and in a phase I clinical trial with intraventricular application of GDNF in patients with ALS, although this study was discontinued because of a lack of efficacy.

In conclusion we have shown widespread RET protein expression in the adult rat spinal cord, including changes in RET expression following nerve injury. The expression and regulation pattern of RET in primary afferent terminals and motoneurons confirms previous findings and is in line with a neurotrophic role of GFLs. The expression of RET in neurons in the dorsal horn that we now show for the first time in detail, supports a role of RET as a mediator of activity-dependent effects of GDNF. We therefore suggest that modulation of GDNF-RET interactions on spinal nociceptive projection neurons may have acute effects on spinal nociceptive transmission. As such, GDNF and RET may be promising targets for the development of pharmaceuticals to alleviate pain and hyperalgesia.

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FIGURES

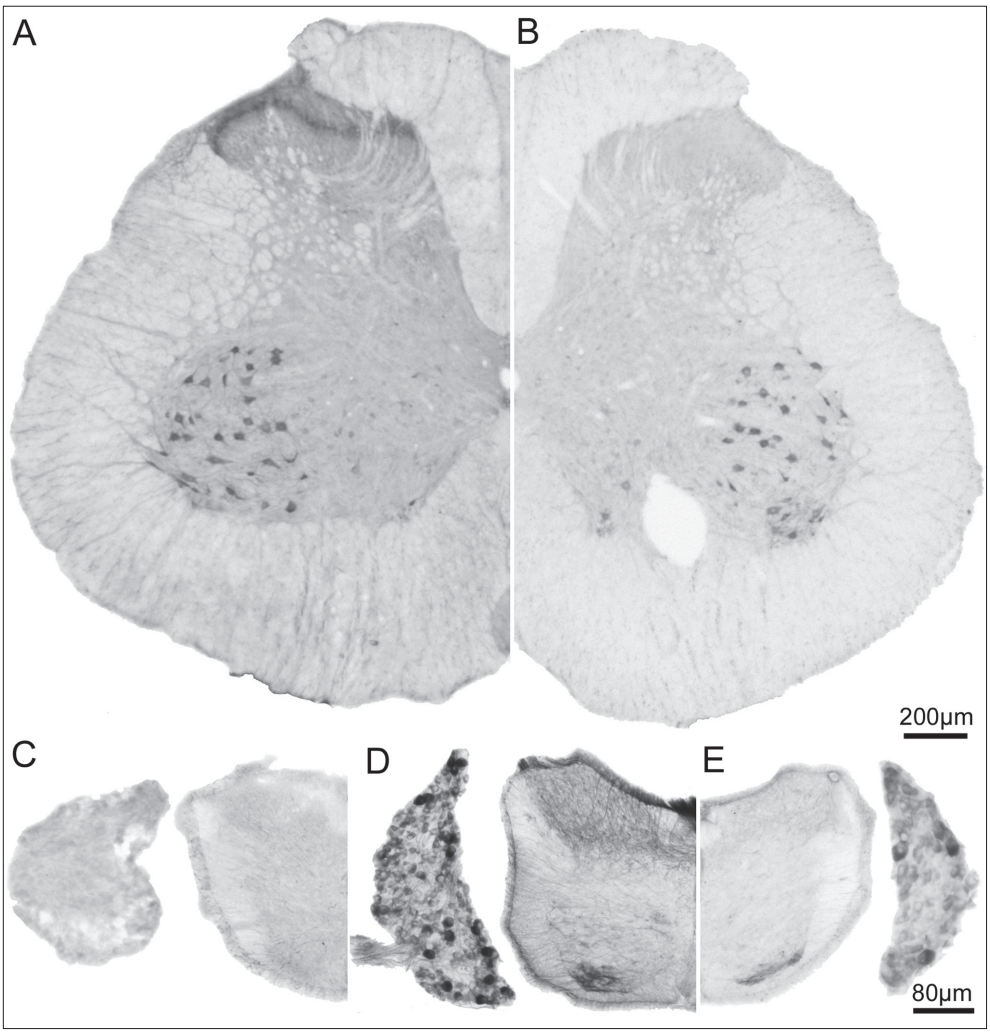


Fig. 1. Light micrograph showing L5 sections from a naïve rat (A, B) and sections from the upper cervical spinal cord with DRGs from a E 17.5 *RET*$^{k-}$ mouse (C) and its wild-type littermate (D, E), all processed for RET-IHC. (A, B) Strong labeling of motoneurons is obvious, but the dense band of immunoreactive fibers in lamina II_i was observed only after pretreatement for epitope unmasking (A). (D, E) Similarly, in embryonic mouse sections RET-ir primary afferent fibers were nearly absent without pretreatment (E), while labeling of motoneurons and DRG neurons was only slightly affected. (C) No neuronal labeling was observed in sections from a *RET*$^{k-}$ mouse.

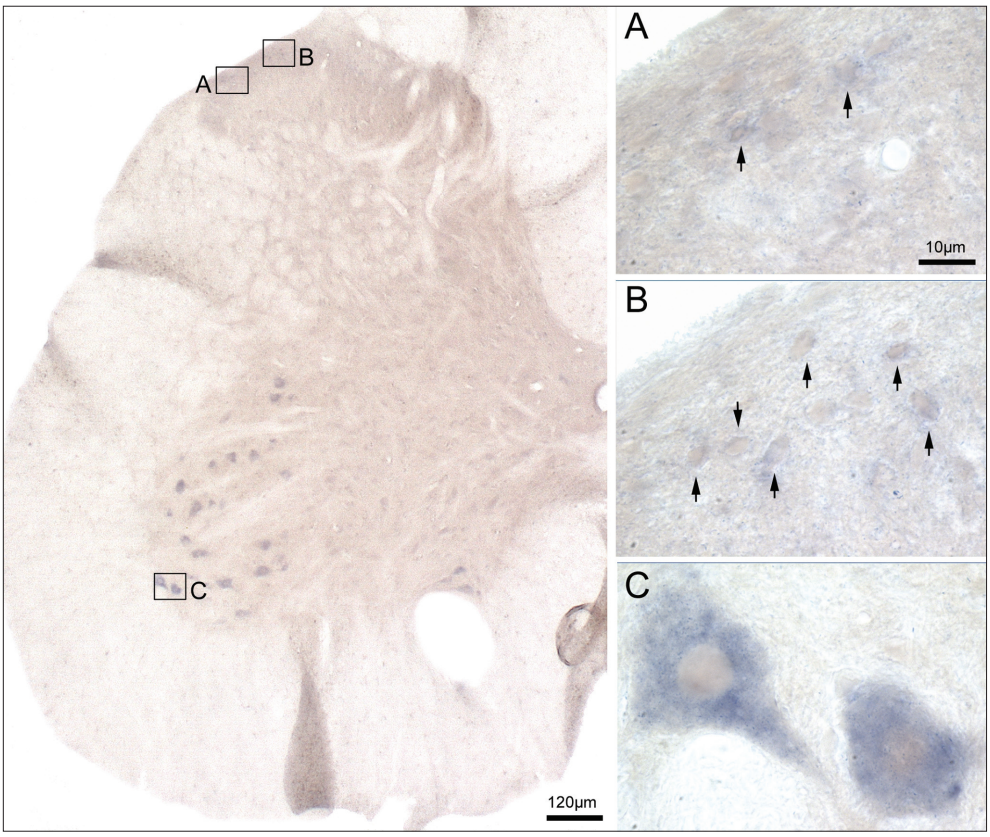


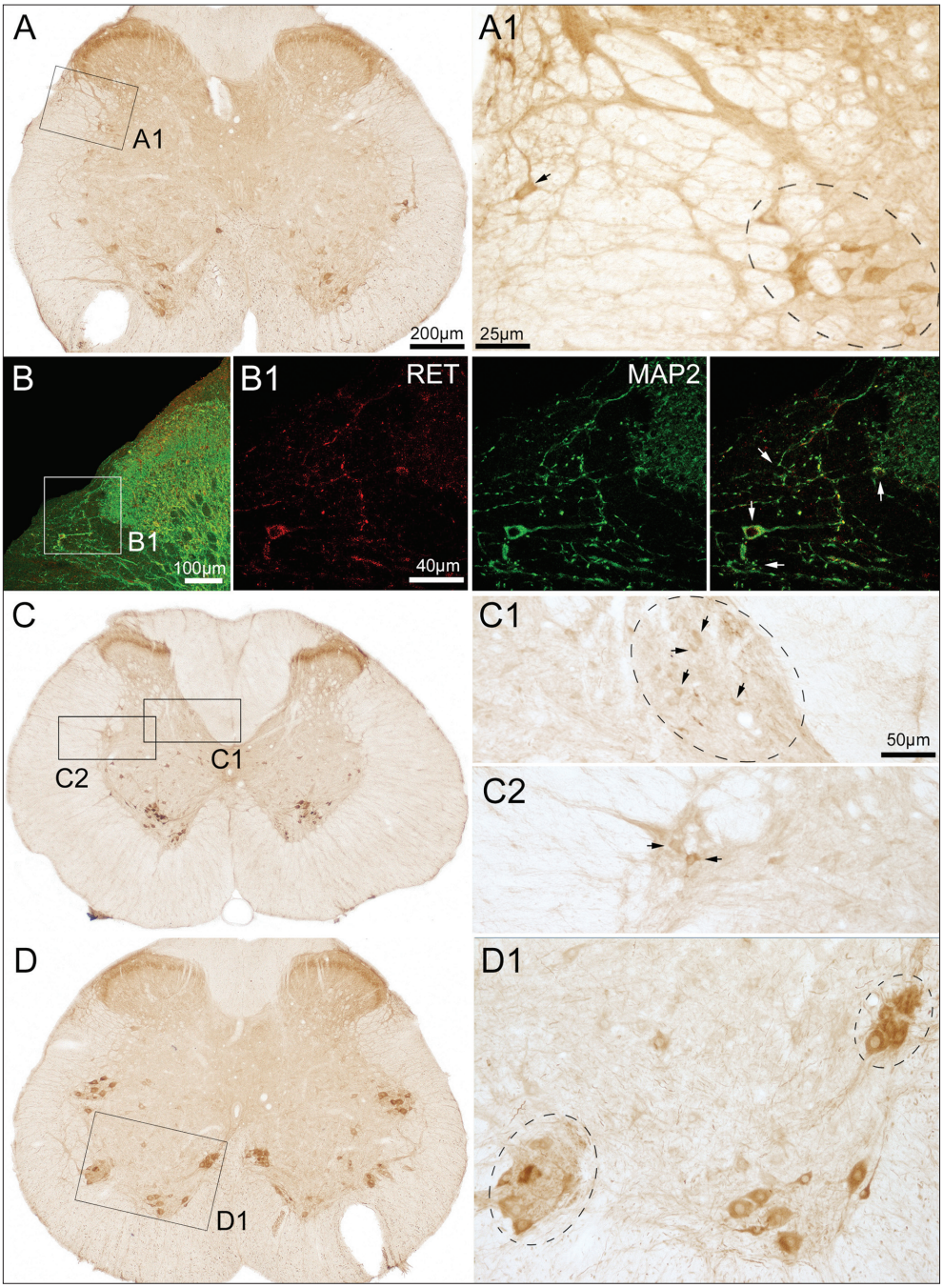
Fig. 2. Light micrograph showing a L4 section from a naïve rat processed for non-radioactive RET-ISH. Strong RET-ir is expressed inmotoneurons (C) and weaker but still distinct labeling is shown in the superficial dorsal horn, as indicated by arrowheads (A,B).

	Labelled structures	Labeling intensity
Lamina I	Neurons	++/+++
Lamina IIo	Neurons	++
Lamina IIi	Primary afferent terminals	+++
Lamina III/IV	Neurons, dendrites	++
Lamina V	Neurons, dendrites	++
Lamina VI, VII, VIII	Neurons, dendrites	+/++
lamina IX	Motoneurons, dendrites	+++/++++
Lamina X	Tanycytes	++
LSN	Neurons	++
DN	Neurons	+
IML	Neurons	+/++
SPN	Neurons	+/++
DM and DL	Motoneurons	+++/++++

Table. Distribution and labeling intensity of RET-ir in the dorsal horn of the adult rat.

Laminae are according to (Molander et al., 1984). Abbreviations: lateral spinal nucleus (LSN), the dorsal nucleus (DN), the intermediolateral nucleus (IML), the sacral parasympathetic nucleus (SPN) and the dorsomedial and dorsolateral nucleus (DM and DL).

Fig. 3. (next page) Light micrographs (A, C, D) and confocal IF images (B) showing sections from naïve rats processed for RET-IHC/IF at different spinal levels. In the confocal images, RET was labeled with Cy3 (red), MAP2 with FITC (green). (A, A1) S2 section showing a RET-ir neuron in the lateral spinal nucleus (arrow) and several neurons in the sacral parasympathetic nucleus (dashed oval). (B) Three RET/MAP2 double labeled neurons and dendrites in the lateral spinal nucleus and one in lamina I (arrows) of a L3 section. Thickness of optical sections: 4.6 μm (B), 1 μm (B1). (C) Several weakly labeled neurons, some of which are indicated by arrows, in the dorsal nucleus (C1) and intermediolateral nucleus (C2) in a L2 section. (D) L6 section showing RET-ir in the dorsomedial and dorsolateral nucleus (D1; dashed ovals). Note: bundles of dendrites characteristically radiating away from the motoneurons in the DM nucleus. All sections but the ones in (B) were treated using the unmasking procedure.


A
A1
200µm
A1
25µm
B
B1
100µm
B1
RET
40µm
MAP2
C
C2
C1
C1
50µm
C2
D
D1
D1

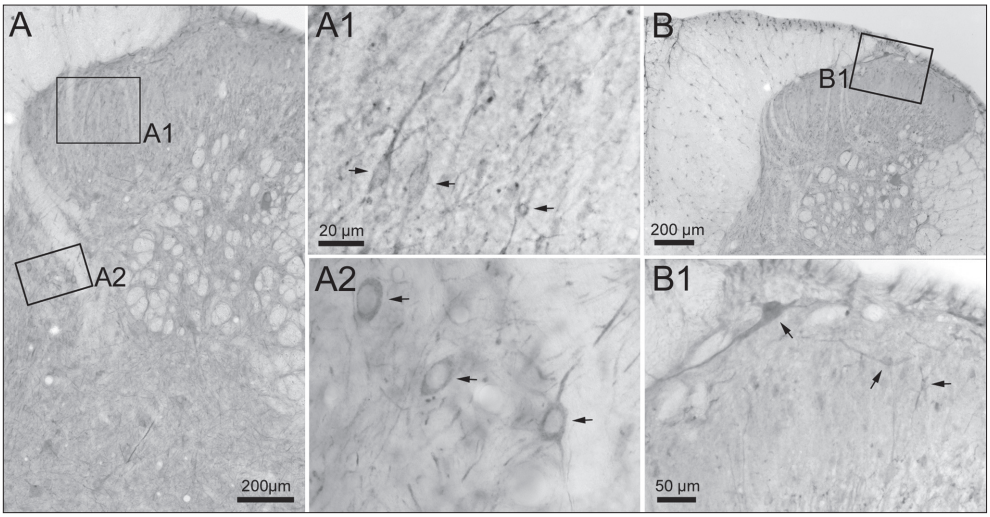


Fig. 4. Light micrographs showing the dorsal horn of a L5 (A) and a L3 (B) section from a naïve rat processed for RET-IHC. RET-ir neurons (arrows) are shown in lamina III (A1), the medial part of lamina V (A2) and in lamina I (B1). RET-ir is preferentially localized in the cytoplasm and primary dendrites of these neurons. These sections were processed without the unmasking procedure, hence labeling of primary afferent fibers is very weak, greatly facilitating the identification of neuronal somata. Figure A1 is an overlay of two optical sections.

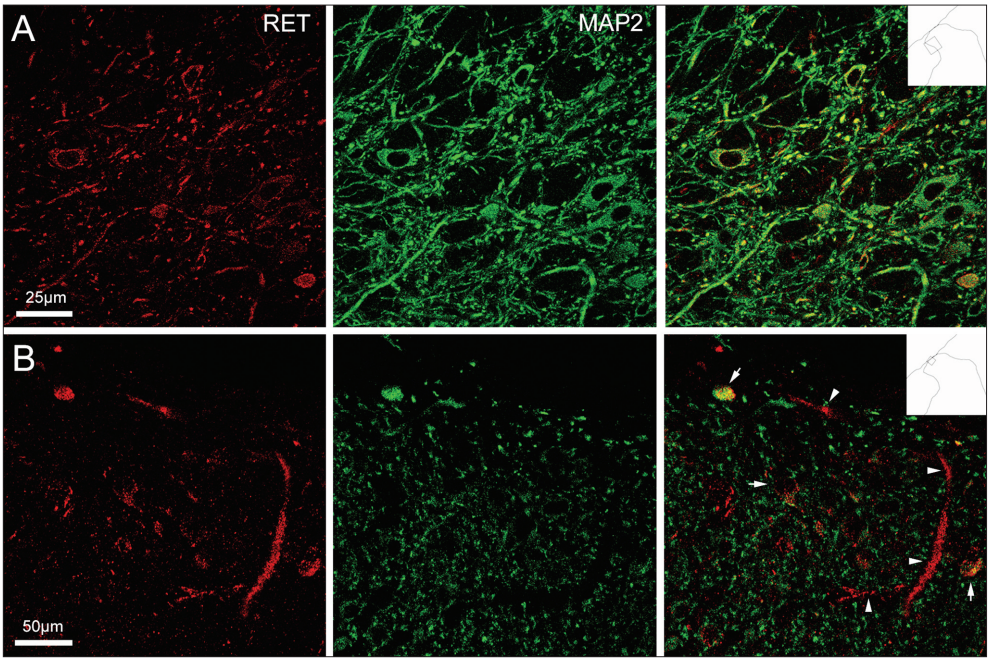


Fig. 5. Confocal IF images of the dorsal horn of an L4 section from a naïve rat processed for RET and MAP2-IF. RET was labeled with Cy3 (red), MAP2 with FITC (green). Overlapping signals are present in cell somata in lamina I and IV (A, B), and in fibers in lamina IV (A). Insets indicate where images were taken. (A) Colocalization of RET and MAP2 confirms the presence of RET in neuronal somata and dendrites. (B) Neuronal somata in lamina I are indicated by arrows. The elongated single labeled structure, indicated by arrowheads, represents an axon. The section was processed without the unmasking procedure. Thickness of optical sections: 1 μm.

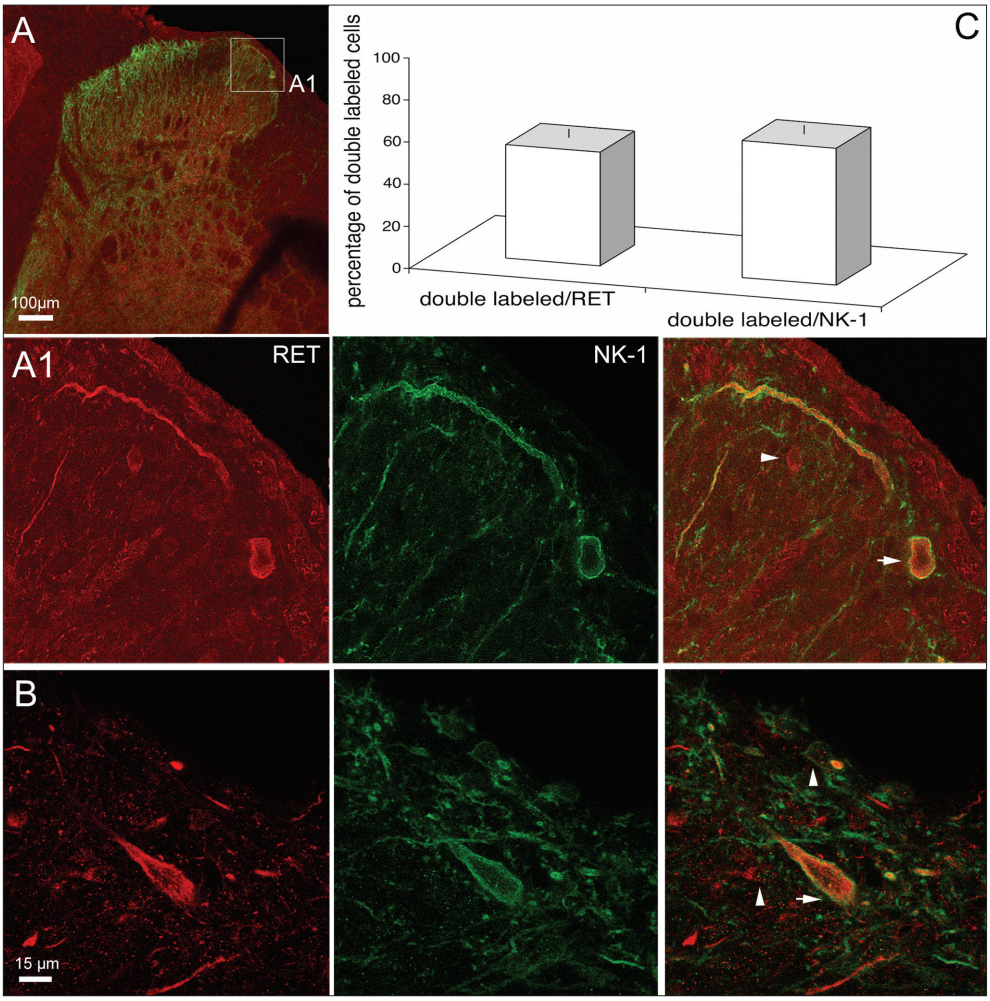


Fig. 6. Confocal IF images of the dorsal horn of an L4 section from a naïve rat processed for RET and NK-1-IF. RET is labeled with Cy3 (red), NK-1 with FITC (green). (A1) A large neuron and dendrite in lamina I are double labeled for RET and NK-1 (arrow). Note preferential localization of RET-ir in the cytoplasm and NK-1-ir alongside the limiting membrane of the soma and the dendrite. The small RET-ir neuron is not double labeled with NK-1 (arrowhead). (B) Large double labeled neuron (arrow) and two single labeled neurons (arrowheads), one RET-ir and one NK-1-ir. The sections were processed without the unmasking procedure. Thickness of optical sections: 4.6 µm (A), 1 µm (A1, C). (C) Histogram, showing percentage of colocalization of RET and NK-1 in lamina I.

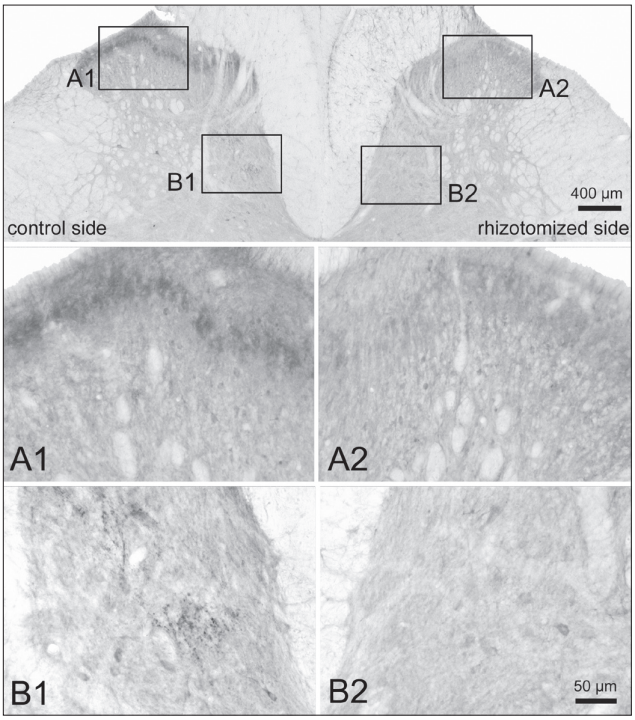


Fig. 7. Light micrographs showing the dorsal horn of a L4 section from a rhizotomized rat, processed for RET-IHC. On the control side strong RET-ir is present in lamina II_i (A1) and RET-ir is expressed in clusters of varicose fibers in the central part of lamina IV and V (B1). On the rhizotomized side RET-ir almost completely disappeared from both superficial (A2) and deep dorsal horn (B2). This section was treated using the unmasking procedure.

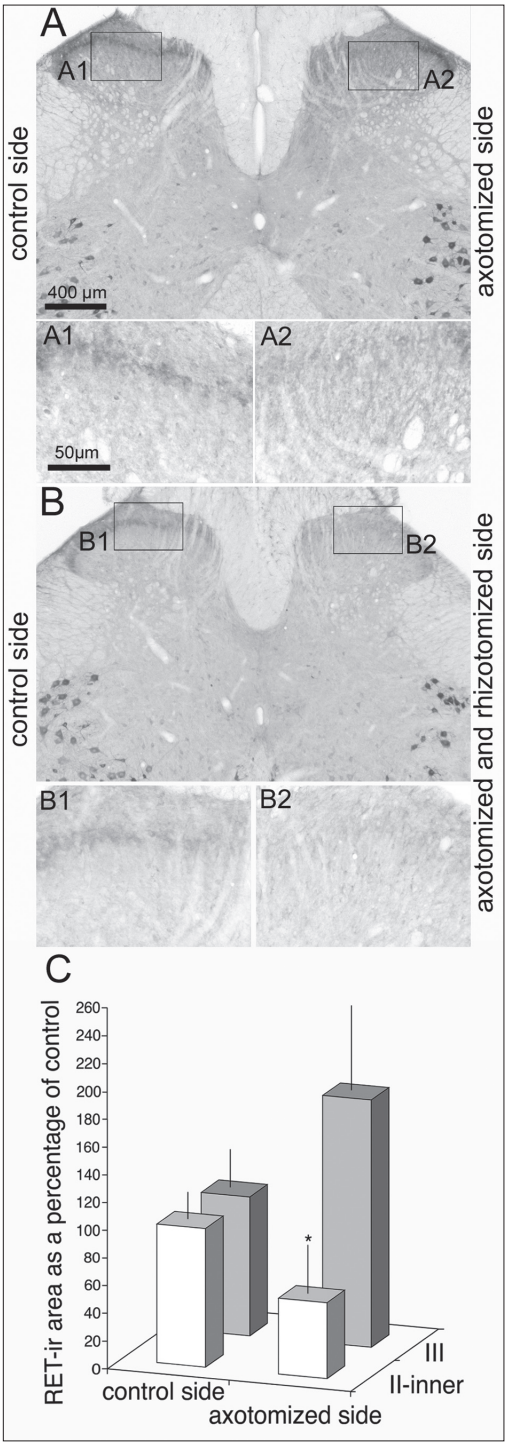


Fig. 8. Light micrographs of L5 sections processed for RET-IHC from a rat after sciatic nerve transection (A) and from a rat in which sciatic nerve transection was followed by dorsal rhizotomy (B). RET-ir is decreased in lamina II_i and apparently increased in lamina III and IV (A2). The apparent increase of RET-ir in lamina III and IV is of primary afferent origin since it disappeared following dorsal rhizotomy (B2). (C) Histogram showing means ± SEM of the area occupied by RET-ir fibers, relative to the control side, which was set at 100%. Significance levels: $*P \leq 0.05$, versus control side, paired *t*-test.

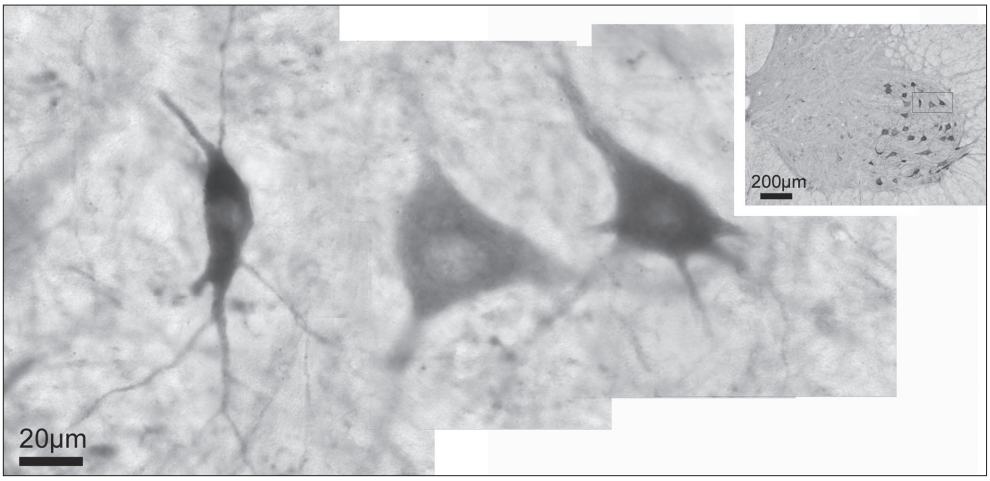


Fig. 9. Light micrograph showing a composition of 20 high power images of a part of the ventrolateral gray matter (see inset) from the same L5 section as in Fig 1. Labeling is present in the cytoplasm and dendrites with sparing of the nucleus. Note that the intensity of RET-ir varies with the size of the motoneurons.

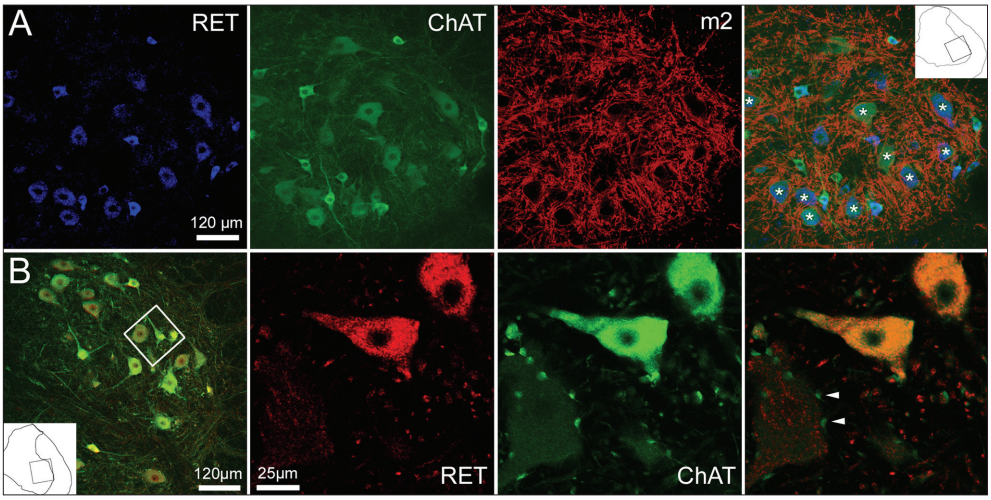


Fig.10. Confocal IF images of the ventral horn of a L4 (A) and a L3 (B) section from a naïve rat processed for RET, ChAT and m2 receptor-IF. Insets indicate where images were taken. (A) RET is labeled with Cy5 (blue), ChAT with FITC (green) and m2 receptor with Cy3 (red). Virtually all ChAT-ir motoneurons also expressed RET. Generally, RET-ir and ChAT-ir is strongest in small size motoneurons. Many medium to large size motoneurons with relatively weak RET-ir expressed m2-receptor on their plasma membrane, the latter indicated by a thin red line that directly surrounds the motoneurons (asterixes). Small motoneurons with strong RET labeling did not or very weakly express m2-receptor. (B) RET is labeled with Cy3 (red), ChAT with FITC (green). A large motoneuron with weak RET labeling receives multiple ChAT-ir boutons on its plasma membrane, two of which are indicated with arrowheads in the merged high-power image. Two small, strongly RET-ir neurons do not receive cholinergic boutons. Thickness of optical sections: 4.6 μm for low-power images, 1μm for high power images. The high-power images in figure B are overlays of a stack of 4 optical sections.

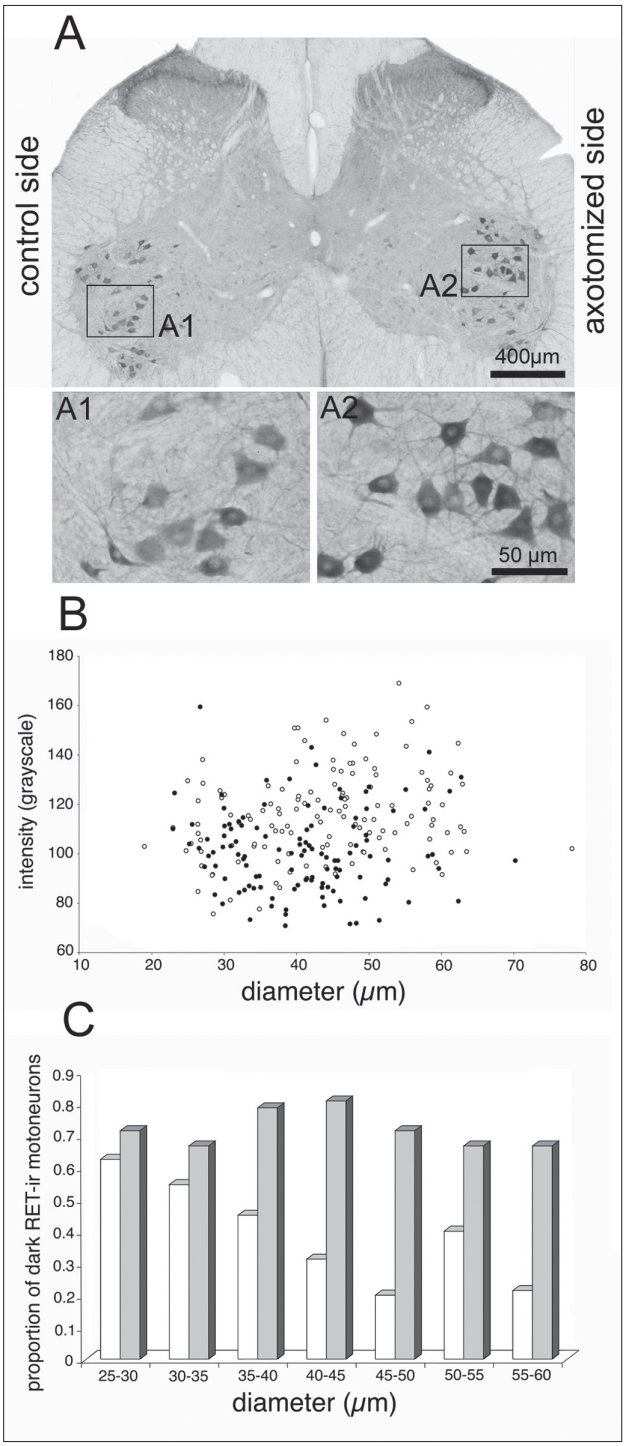


Fig. 11. (A) Light micrograph of a L5 section from a rat after sciatic nerve transection, processed for RET-IHC. Sciatic nerve transection induced a strong increase in the proportion of darkly stained motoneurons. This increase was almost entirely due to increased staining of large diameter motoneurons. Note that the increase only occurred in the motoneuron pool contributing to the sciatic nerve. (B) Scatterplot relating neuronal size to RET staining intensity on the control (open circles) and axotomized side (closed circles) of rats after sciatic nerve transection. Generally neurons on the axotomized side were darker than those on the control side. (C) Size-proportion histogram showing means ± SEM of the proportion of motoneurons with staining intensities lower than 110 on 256 level gray scale, i.e. darkly stained motoneurons. The increase in proportions of darkly stained motoneurons on the axotomized side (gray bars) versus the control side (white bars) was statistically highly significant: $P \leq 0.01$, axotomized versus control side, paired t-test.

Chapter 6

General discussion

In this thesis, a series of experiments were described and in each chapter the results were discussed in detail. All these experiments suggest that Glial cell line-Derived Neurotrophic Factor (GDNF) plays a role as a neuromodulator in spinal pain transmission and pieces of evidence for this suggestion were brought forward in each of the chapters. Here, in the general discussion, all the evidence that now exists on the issue whether or not GDNF plays a neuromodulatory role in the spinal dorsal horn, will be reviewed. Finally, the potential relevance of the findings in this thesis for clinical practice will be discussed.

MAY GDNF BE CONSIDERED A NEUROMODULATOR IN SPINAL PAIN TRANSMISSION?

In order to answer this question, it must first be determined what is meant by the term neuromodulator. This question is difficult to answer, since no clear definition exists. Originally the term neuromodulator took root when it was discovered that next to the classical neurotransmitters, like the amino acids and monoamines, neuropeptides could be present in the same terminal, "modulating" the postsynaptic effect of classical neurotransmitters (Hokfelt et al., 1980; Hokfelt et al., 1975). Nowadays, the term neuromodulator is generally used to indicate substances that are co-released with the classical neurotransmitters, although they may have different postsynaptic effects, which are usually mediated through activation of G-protein coupled receptors or tyrosine kinase receptors. Since no definite criteria for the term neuromodulator exist, the criteria that define a neurotransmitter will be used for evaluating GDNF, also because there are no compelling arguments to suggest that these criteria should not be applied.

Criteria for neurotransmitters

The criteria for considering a particular substance as a neurotransmitter have evolved slowly in the past century. In 1921, acetylcholine, then termed “Vagusstoff”, i.e. a substance originating from the vagus nerve, was the first substance to be identified as a chemical transmitter, by Otto Loewi (Fig. 1). Since then numerous other transmitter-like substances were identified, which according to some should be considered as neurotransmitters, while others argued that there was not enough evidence to make such a claim. From this discussion a set of criteria emerged that had to be fulfilled if a substance was to be considered a neurotransmitter. The criteria for a putative neurotransmitter may be summarized as follows (Pezet et al., 2002; Purves, 2004):

1. A putative neurotransmitter should be synthesized within a neuron and the substance should be found within a nerve terminal.

2. It should be released following stimulation.

3. After release it should act on a post-synaptic receptor and cause a biological effect.

4. When the putative neurotransmitter is applied on the post-synaptic membrane, it should have the same effect as when it is released by a neuron. Known antagonists of the putative neurotransmitter should block its effect in a dose-dependent manner.

5. After a putative neurotransmitter is released from a neuron, it should be inactivated, e.g. through a reuptake mechanism or by an enzyme that stops the action of the transmitter.

We will now consider to what extent GDNF also meets the above criteria in the context of nociceptive transmission in the dorsal horn of the spinal cord.

The first criterion states that a putative transmitter should be synthesized within a neuron and that it should be found within a nerve terminal. To comply with this criterion in the context of spinal nociception, GDNF should be synthesized in dorsal root ganglion (DRG) cells and it should be found within primary afferent terminals in the spinal dorsal horn. Several authors have described GDNF protein, identified by immunohistochemistry, in the somata of DRG neurons (Chapter 2 and Bar et al., 1998; Dong et al., 2005a; Dong et al., 2005b; Fang et al., 2003; Honda et al., 1999; Ohta et al., 2001) and those of the trigeminal ganglion (Del Fiacco et al., 2002; Quartu et al., 1999). Specificity of the antibody was confirmed by standard immunohistochemical controls (Chapter 2) and also by clearly enhanced Western blot levels in mice overexpressing GDNF (Meng et al., 2000). However, labeling of cell somata is relatively weak in comparison to fiber and terminal labeling in the superficial dorsal horn (Chapter 2 and Ohta et al., 2001). The presence of GDNF protein suggests that GDNF is synthesized in the DRG neuron, but clearly identification of GDNF mRNA is necessary to prove unambiguously that GDNF is actually synthesized in these neurons. Sofar GDNF mRNA has not been detected in studies that included DRG neurons in their general description of GDNF mRNA in the nervous system (Golden et al., 1998; Hammarberg et al., 1996; Josephson et al., 2001; Widenfalk et al., 1999). Therefore some authors (Rind and von Bartheld, 2002; von Bartheld et al., 2001) have suggested that GDNF protein in DRG neurons originates from sources outside the DRG, i.e. Schwann cells or target tissues. If this holds true, the presence of GDNF protein would be explained by uptake and retrograde transport of GDNF protein synthesized by Schwann cells or peripheral tissues. Since GDNF protein is abundant in the terminals of primary afferent fibers in the dorsal horn, this would imply that, after retrograde transport from the periphery, GDNF protein is transported anterogradely towards the terminals in the spinal cord for release through a process called neuronal transcytosis (Rind and von Bartheld, 2002; von Bartheld et al., 2001). An alternative and more plausible possibility is that the GDNF mRNA levels are too low to be detected by standard *in situ* hybridization procedures. This is supported by the fact that GDNF immunoreactivity is clearly enhanced by

pretreatment of animals with colchicine, which blocks axonal transport (Ohta et al., 2001). If GDNF protein would originate in the periphery, an axonal transport block would also block the flow of GDNF protein from the periphery towards the DRG somata, leading to unchanged levels of GDNF in the DRG (or a decrease due to breakdown of GDNF protein) rather than an increase in GDNF. In contrast, the observed increase in GDNF immunoreactivity favors an accumulation of GDNF that is synthesized in DRG somata. Furthermore, ligation of the sciatic nerve in rats led to an accumulation of GDNF at the ganglion side of the ligation (Neuteboom, R.F. and Holstege, J.C., unpublished observation and Honda et al., 1999), indicating that GDNF is transported anterogradely (i.e. away from the DRG soma) rather than retrogradely. Finally, it may be argued that the weak GDNF immunohistochemical signal in the DRG somata indicates that GDNF, after synthesis, is directly transported peripherally to the terminals in peripheral tissues and centrally to terminals in the spinal dorsal horn, where it accumulates. This would also explain weak somatic and strong terminal labeling.

GDNF is present in fibers that terminate in lamina I and the outer layer of lamina II of the spinal dorsal horn (Chapter 2) and (Ohta et al., 2001). These primary afferent fibers are the only source of GDNF in the dorsal horn, since a transection of these fibers (a dorsal rhizotomy) leads to a complete disappearance of GDNF from the dorsal horn (Chapter 2). Based on the immunohistochemical distribution of GDNF in the spinal cord, which is almost identical to the distribution of the peptidergic primary afferent fibers, in many of which substance P, CGRP and galanin are colocalized, we suggest that GDNF is also localized in neurons belonging to the subgroup of peptidergic primary afferents. This idea is substantiated by the finding that GDNF colocalizes with SubP in the spinal cord (Fang et al., 2003). Furthermore, it was demonstrated that GDNF protein is mainly present in small to medium sized DRG neurons and that only a small number of GDNF-ir DRG cells belonged to the group of non-peptidergic neurons (Honda et al., 1999). All these findings are in accordance with a localization of GDNF in fibers from peptidergic neurons.

Ultrastructural studies of the localization of GDNF in the superficial dorsal horn showed that GDNF was localized in dense-cored vesicles (Holstege et al., 1999; Ohta et al., 2001) (Fig. 2). This type of vesicle also contains neuropeptides like Substance P (SubP), calcitonin gene-related peptide (CGRP) and the neurotrophin Brain-Derived Neurotrophic Factor (BDNF). Dense-cored vesicles are filled with neuropeptides in the Golgi complex in the DRG soma and subsequently transported towards the axon terminals where they are released in an activity dependent manner, but only when calcium levels in the terminal are relatively high, i.e. after strong and prolonged stimulation. This suggests that GDNF is released in a similar, activity dependent, way. In conclusion, the presence of GDNF protein in DRG somata, the anterograde transport within primary afferent fibers and its localization in dense-cored vesicles in terminals of primary afferents in the superficial dorsal horn, are all in agreement with synthesis of GDNF within DRG neurons and its presence in primary afferent terminals.

The second criterion states that a putative neurotransmitter should be released following stimulation. With respect to GDNF, there is presently no direct evidence suggesting that GDNF is released in an activity dependent manner. The main argument here is that GDNF is localized within dense-cored vesicles, which implies that GDNF is released the same way as dense-cored vesicles release their content. In contrast to the release from small clear vesicles, release from dense-cored vesicles occurs not only at the active zones in nerve terminals, but also at extrasynaptic sites (Purves, 2004). Furthermore, release from dense-cored vesicles only takes place when there is a significant rise in the calcium level throughout the terminal, which is achieved solely by high frequency stimulation, i.e. by strong nociceptive stimulation. So far GDNF release from neurons has never been shown *in vivo* or in an *ex-vivo* dorsal root-spinal cord preparation, like has been shown for BDNF (Lever et al., 2001). Actual release has only been shown *in vitro* from neural progenitor cells that were genetically modified to secrete GDNF (Behrstock et al., 2005; Klein et al., 2005), which were intended for the treatment of amyotrophic lateral sclerosis and Parkinson's disease. In conclusion, only the above discussed indirect evidence suggests that GDNF is released following stimulation.

The third criterion states that, following release, a putative neurotransmitter should act on a post-synaptic receptor and cause a biological effect. GDNF signaling is mediated by a receptor complex consisting of RET, the signal transducing element, and GFRα-1, the GDNF binding co-receptor. The presence of GFRα-1 has been demonstrated in the spinal dorsal horn using *in situ* hybridization (Glazner et al., 1998; Widenfalk et al., 2001) and immunohistochemistry (Matsuo et al., 2000). In Chapter 4 and 5 of this thesis it was shown that RET is expressed in dorsal horn neurons, that were identified by their simultaneous expression of the neuron specific marker MAP2 (Chapter 5). RET expressing neurons in lamina I at least partly represent nociceptive specific projection neurons, both by morphology (chapter 3) and by a high degree of colocalization with the SubP receptor NK-1 (chapter 4). In conclusion, in the termination area of GDNF containing primary afferent fibers, many (nociceptive specific) RET and GFRα-1 neurons are present, showing that there is a good match between the putative neurotransmitter (i.e. GDNF) and its postsynaptic receptor (i.e. RET and GFRα-1). This provides the basic requirements for GDNF to act as a neuromodulator in spinal pain transmission.

The fourth criterion states that when a putative transmitter is applied on the post-synaptic membrane, it should have the same effect as when it is released by a neuron, and known antagonists of the putative transmitter should block its effect in a dose-dependent manner. In 2001 it was shown for the first time that GDNF induced acute effects, namely the activation of potassium channels on dopaminergic cells *in vitro*, via a mechanism that involves activation of MAP kinase (Yang et al., 2001). Later it was shown that this effect was not restricted to potassium channels but also involved acute potentiation of calcium channels (Wang et al., 2003). Chapter 3 describes that intrathecal GDNF injection in rats rapidly induces an upregulation of the

immediate early gene (IEG) products c-Fos, c-Jun and Krox-24 in spinal dorsal horn neurons, which effect was concentration dependent and, in case of c-Fos, occurred in about 40% of RET expressing neurons. In conclusion, rapid postsynaptic effects of exogenously applied GDNF favor a role as a neuromodulator, although additional experiments using GDNF antagonists are urgently needed to see whether the rapid effects from exogenous GDNF parallel those from endogenous GDNF release in response to physiological stimuli.

The fifth criterion states that after a putative neurotransmitter is released from a neuron, it should be inactivated, e.g. through a reuptake mechanism or by an enzyme that stops the action of the transmitter. In order to satisfy this criterion for GDNF in the context of spinal nociception there must be a mechanism in place, which terminates its actions, following release from primary afferent terminals in the dorsal horn. Generally, there are three ways in which GDNF may be inactivated after release. Firstly, GDNF may simply diffuse away from the site of release and become functionally inactive by dilution, which was actually shown in Chapter 3. Secondly, GDNF's actions may be terminated by uptake from soluble GFRα-1. Soluble GFRα-1 is involved in so called *in-trans* signaling (Paratcha and Ibanez, 2002) (Chapter 5), which is thought of as a way to increase sensitivity for GDNF signaling (Paratcha et al., 2001). Alternatively it may speculated that these soluble receptors contribute to removal of GDNF from synapses, just as it has been suggested that BDNF may be inactivated by truncated Tyrosine kinase B (TrkB) receptors (Pezet et al., 2002). Finally, GDNF may be inactivated by enzymatic degradation. Although such a mechanism is commonly accepted for small molecule neurotransmitters such as acetylcholine, it is not regarded a general principle for neuropeptides and proteins like BDNF (Pezet et al., 2002). In conclusion, although inactivation through diffusion has been shown for exogenously applied GDNF and this may fit its role as a neuromodulator, it is currently not clear which mechanisms contribute to endogenously released GDNF inactivation.

When reviewing the criteria as they were applied to GDNF, it may be concluded that evidence so far is consistent with a role of GDNF as a neuromodulator in spinal pain transmission and, equally important, that none of the data argue directly against a neuromodulator role of GDNF. However, additional data are urgently needed to make this claim unambiguously. Firstly, detection of GDNF mRNA is necessary to prove synthesis of GDNF in DRG neurons. Secondly, actual release of GDNF from primary afferent terminals in response to nociceptive stimuli and demonstration of biological effects from endogenously released GDNF is another prerequisite to firmly establish GDNF as a neuromodulator in spinal pain transmission.

IS GDNF AN ANALGESIC OR A HYPERALGESIC COMPOUND?

Neurotrophic factors have different roles in the nervous system. They may act as trophic factors, in which case they are taken up from the environment and transported retrogradely towards the cell soma to exert their effects on the neuron. Alternatively, they may be produced by the neuron, transported anterogradely towards the nerve terminal and be released to exert their effects as a transmitter on the next neuron. As stated in the introduction of this thesis, the trophic effect of neurotrophic factors is evident from its role in survival and differentiation of neurons during development and its regenerative effects on damaged neurons in adulthood. Thus, exogenous application of NGF and BDNF can reverse pathological changes that occur after damage to nociceptive fibers, by virtue of their retrograde trophic actions. Since neuropathic pain is one of the main consequences of damage to nociceptive fibers, it is not surprising that NGF and BDNF have been shown to reverse neuropathic pain behavior (Apfel et al., 1994; Apfel et al., 1991; McArthur et al., 2000; Mitsumoto et al., 1994; Ren et al., 1995). NGF and BDNF may therefore be considered analgesic factors in neuropathic pain, by virtue of their capacity to counteract the pathological hyperalgesic effects that occur after nerve lesion.

In inflammatory conditions, the roles of NGF and BDNF are entirely different from those in neuropathic pain. In this case, when there is no damage to nociceptive fibers, NGF is released from peripheral inflammatory cells, acting as a pro-inflammatory cytokine, and is both necessary and sufficient for the induction of hyperalgesia (Shu and Mendell, 1999). BDNF, produced by spinal ganglion cells and released from their terminals in the spinal cord, acting as a neurotransmitter, also induces inflammatory hyperalgesia (Zhao et al., 2006). NGF and BDNF may therefore be considered hyperalgesic factors in inflammatory pain, considering their net effect on nociceptive transmission.

A similar situation applies to the role of GDNF in nociception. GDNF determines the phenotype of primary afferent neurons in the early postnatal period and acts as a survival factor for axotomized nociceptors in adult animals. It was shown that intrathecal application of GDNF reverses phenotypical changes induced by peripheral axotomy (Bennett et al., 1998) and neuropathic pain behavior (Boucher et al., 2000), by virtue of its trophic effects. Thus, GDNF may be considered an analgesic factor in neuropathic pain. In contrast to the trophic effects of GDNF in pathological situations like neuropathic pain, we have provided evidence in this thesis that the physiological role of GDNF during adulthood is that of a neuromodulator involved in spinal nociceptive transmission. Supposing such a role for GDNF, the question immediately arises whether GDNF in these situations acts as an analgesic or a hyperalgesic compound? In chapter 4 of this thesis we have demonstrated increased c-Fos expression in superficial dorsal horn neurons that express RET, the receptor for GDNF, following intrathecal GDNF injection. C-Fos is considered a marker of neuronal excitation and such an excitatory effect of GDNF on spinal neurons is in line with excitatory effects of GDNF elsewhere in the nervous system (Wang et al., 2003; Yang et al.,

2001). Since in lamina I RET colocalizes with NK-1 to a high degree and since the majority of neurons in lamina I are known to be nociceptive specific neurons, increased c-Fos expression in SDH neurons following GDNF injection suggests a hyperalgesic action for GDNF. In a rat model of arthritis pain, an intrathecally applied GDNF function-blocking antibody resulted in attenuation of pain behavior (Fang et al., 2003). However it was also postulated (Malcangio et al., 2002) that GDNF has an analgesic effect during inflammation, through an indirect effect on dorsal horn somatostatin release.

It may be concluded that the findings on the effects of GDNF regarding the modulation of nociceptive information in the spinal cord are limited and inconclusive. Based on the findings provided in this thesis, it seems most likely that GDNF exerts an excitatory effect on nociceptive neurons, in other words GDNF acts as a hyperalgesic transmitter in the spinal cord. Further experiments are needed to substantiate this conclusion.

FROM EXPERIMENTAL NOCICEPTION TO CLINICAL PAIN

As stated in the introduction where the organization of the nociceptive system was discussed, most of our knowledge regarding pain relates to the initial stages of pain processing, while the later stages, including the affective-motivational aspect of pain, have only recently begun to be explored, with the help of functional MRI and other imaging techniques. Furthermore, by far the greatest part of our knowledge of the pain system is based on experiments using cells, tissues and laboratory animals. So the question soon arises, what can cells, tissues and animals teach us about pain processing and the treatment of pain? The answer is: a lot, but not everything. To illustrate this, three examples of advances in the treatment of pain and how they relate to basic research will be discussed.

The leaves from willow trees were already known to have analgesic efficacy to animals and to the ancient Greeks when the active ingredient became commercially available to humans as Aspirin® in 1899. However, it was not until the 1960s-1970s that its exact mechanism of action became known. Aspirin® turned out to be an inhibitor of the cyclo-oxygenase (Cox) enzyme, which is responsible for the conversion of arachnidonic acid into prostaglandins, which are powerful proinflammatory agents. The experimental work leading to this discovery was awarded with the Nobel prize in Medicine or Physiology for Bergstrom, Samuelsson and Vane in 1982 (Oates, 1982) and stimulated pharmaceutical companies to develop new Non-Steroidal Anti Inflammatory Drugs (NSAIDs) and later, following the discovery of two different Cox-enzymes (Hla and Neilson, 1992), a whole new group of analgesics, called the Cox inhibitors (Coxibs). The second example is opium, which has been used as a treatment for pain since ancient times in different cultures. In the early 19th century morphine was extracted as the single analgesic compound in the poppy. Although

most of our knowledge regarding the clinical pharmacology of opioids results from experiments on humans and clinical observations, the discovery of opioid receptors in guinee-pig gut in the 1960s (Gyand and Kosterlitz, 1966) and the subsequent discovery of the periaqueductal grey (Tsou and Jang, 1964) and spinal cord (Gouarderes et al., 1985; Yaksh and Rudy, 1976) as sites of action of morphine in rats spurred a large amount of basic experimental work that led to the development of new pharmacological compounds with various affinities for the three opioid receptors, such as oxycodone and fentanyl, and the application of novel techniques of analgesia, such as spinal analgesia. The third example is the identification of the anterolateral system and more recently the dorsal column pathway for visceral pain (Willis et al., 1999) in animals, that paved the way for new treatments in cancer patients. Nowaydays cordotomy for intractable somatic pain and midline myelotomy for visceral pain in patients with advanced cancer has become a standard procedure in tertiary cancer centers.

Thus, experimental pain research has contributed tremendously to the development of analgesics and physical analgetic treatments. However, there are still many conditions which are refractory to current pain treatment, the most obvious being cancer pain and neuropathic pain. Certainly, animal models of cancer pain (Halvorson et al., 2005; Medhurst et al., 2002; Schwei et al., 1999) and neuropathic pain (Bennett and Xie, 1988; Decosterd and Woolf, 2000; Kim and Chung, 1992) may reveal underlying mechanisms of these pain states, but unraveling a particular pain mechanism holds no promise that this will result in an effective treatment. It is well known that new treatments that were effective in experimental animals ultimately failed in clinical trials. There are several explanations for this: firstly, a particular pain inducing mechanism is almost never the simple cause of a pain syndrome. Usually multiple redundant mechanisms act in concert and intervening in one mechanism will be compensated by another. Secondly, it is virtually impossible to assess spontaneous pain in experimental animals, basically because animals cannot communicate their experiences in much detail. Pain in animals is generally assessed by measuring changes in pain thresholds, i.e. evoked pain and not spontaneous pain. In the clinic and outpatient clinic, however, patients complain mostly about their spontaneous pain. Thirdly, it is more and more acknowledged that the affective-motivational aspect of pain has an important contribution to the overall pain experience. In clinical pain trials nowadays therefore pain unpleasantness (Gracely and Dubner, 1987), affective pain scores (Richter et al., 2005) and mood (Lesser et al., 2004) are considered equally important outcome measures as pain intensity. This quality of pain is hard to determine in experimental animals.

How do the above contemplations relate to the current thesis on the involvement of neurotrophic factors in spinal pain transmission? It is true that so far all clinical trials with delivery of neurotrophic factors to patients with diabetic neuropathy (Apfel et al., 1998), amyotrophic lateral sclerosis and Parkinson's disease (Nutt et al., 2003; Gill et al., 2003; Lang et al., 2006) have not shown any clinical efficacy. Therefore one may conclude that the clinical application of

neurotrophic factors is not worth pursuing (Apfel, 2002), but not before potential causes for failure of neurotrophic factors have been critically evaluated. One possible explanation is a practical one: since neurotrophic factors are large molecules subject to degradation and poor penetration, they may not reach their targets during systemic or intrathecal application and novel routes of administration have to be developed to explore their full therapeutic potential. A second reason relates to the fact that no clear distinction between the trophic effects and the rapid modulatory effects of neurotrophic factors were made. So far, clinical trials were initiated to counteract neurodegenerative conditions. However, neurotrophic factors do not act exclusively as trophic factors but may also act as neuromodulators as is argued in this thesis. NGF is now generally accepted as an important mediator of inflammatory hyperalgesia, BDNF as a neuromodulator in spinal nociceptive transmission and GDNF may be added to this list. Based on sound evidence from numerous experimental animal studies showing a strong effect of NGF antagonists in both inflammatory (Lewin et al., 1993; McMahon et al., 1995) and cancer pain (Halvorson et al., 2005; Sevcik et al., 2005), a phase I clinical trial has recently been started to evaluate the efficacy of intraarticular injection of a monoclonal antibody against NGF in osteoarthritis. Thus, based on experimental studies with neurotrophic factors, it becomes clear that the clinical application of neurotrophic factors or its antagonists should be aimed at their fast modulatory effects rather than at their trophic effects. It is true that neurotrophic factors are just one of many classes of substances that are involved in pain sensation. However they constitute a unique class since they act on tyrsine kinase receptors, which may be targeted without affecting the other classes of transmitters and receptors involved in pain transmission. So far compounds targeting tyrosine kinase receptors are used in oncology and pharmaceutical companies will undoubtedly expand application of thesekinds of compounds for use in pain conditions, e.g. when anti-NGF trials will prove successful. In this way research on neurotrophic factors in xperimental animals may not only give insight in important pain mechaniss, but may also identify new target for the treatment of chronic pain.

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FIGURES

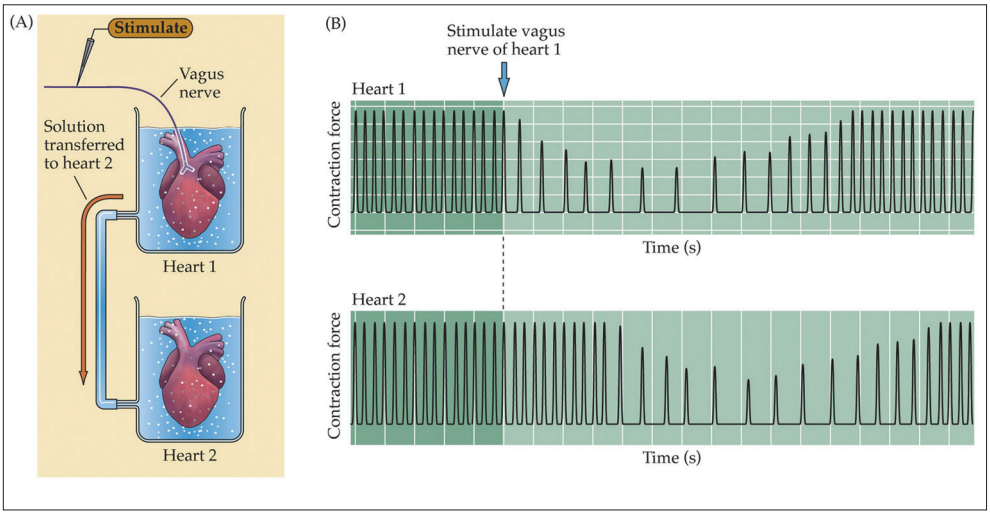

Fig. 1. Otto Loewi's experiment: Two frog hearts were perfused with and kept in warm Ringer's solution. In this condition the hearts continued to beat for a couple of hours. He then stimulated the vagus nerve to heart #1. As a consequence there was a strong inhibition in this heart beats. When the perfusion fluid of heart #1 was transferred to heart #2, exactly the same effect was achieved. Loewi rightly concluded that this must be due to a chemical released from the vagus nerve into the perfusion fluid, which he called "Vagusstoff".

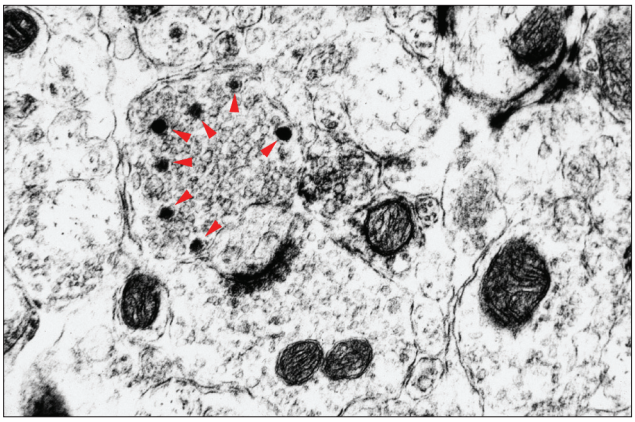

Fig. 2. Electron-microscopical image showing dense-cored vesicles (arrowheads) containing GDNF immunoreactive product in a primary afferent terminal in the superficial dorsal horn of an adult rat.

Chapter 7

Summary / Samenvatting

SUMMARY

The aim of this thesis was to investigate the involvement of glial cell line-derived neurotrophic factor (GDNF) in spinal pain transmission. In Chapter 1 some recent developments regarding the organization of the nociceptive system are discussed, followed by a description of the involvement of neurotrophic factors in sensory transmission. This information provides the necessary background for the interpretation of the findings in the chapters to follow.

In Chapter 2 immunocytochemistry was used to identify GDNF in rat spinal cord. Strong GDNF labeling was found in fibers and terminals in laminae I and II_{outer} and to a lesser extent in the remaining laminae. A few spinal ganglion cells also contained GDNF. After dorsal root transection GDNF disappeared from the dorsal horn and after dorsal root ligation there was accumulation of GDNF only on the ganglion side of the ligation. These findings demonstrate anterograde transport of GDNF within primary afferent fibers, which constitute the only source of GDNF labeling in the dorsal horn. The strong presence of GDNF in the superficial dorsal horn may indicate that GDNF has a role in pain transmission in the adult rat spinal cord.

In Chapter 3 the effect of sciatic nerve axotomy on the expression of GDNF protein in the dorsal horn was investigated, using immunohistochemistry. Image analysis showed a 44% decrease relative to the non-transected side after 5 days survival, progressing to more than 80% decrease after 10 days and remaining so for at least 100 days. This rapid and strong decrease suggests active downregulation of the expression of GDNF protein after peripheral axotomy. The observed downregulation of GDNF is compared with changes observed for other substances in primary afferents after peripheral axotomy and is discussed in light of its presumed trophic or transmitter role in nociception.

In Chapter 4 the effect of GDNF and brain-derived neurotrophic factor (BDNF) injected by lumbar puncture, on the expression of the immediate early gene (IEG) products c-Fos, c-Jun and Krox-24 in the adult rat dorsal horn, was investigated. In the dorsal horn of S1 spinal segments, GDNF and BDNF induced a strong increase in IEG expression, which was most pronounced in laminae I and II (2.9-4.5 fold). More distal from the injection site, in the dorsal horn of L1/L2 spinal segments, the increase in IEG expression was less pronounced, suggesting a concentration-dependent effect. In order to explain the effects of intrathecally injected GDNF, we investigated whether lumbo-sacral dorsal horn neurons expressed RET protein, the signal-transducing element of the receptor complex for GDNF. It was found that several of these neurons contained RET immunoreactivity and that some of the RET-immunoreactive neurons had the appearance of nociceptive specific cells, confirming their presumed role in pain transmission. These results demonstrate that intrathecally applied GDNF and BDNF induce IEG expression in dorsal horn neurons in the adult rat, supposedly by way of their cognate receptors, which are present on these neurons. It is suggested that the endogenous release of GDNF and BDNF, triggered by nociceptive

stimuli, is involved in the induction of changes in spinal nociceptive transmission as in various pain states.

RET (REarranged during Transfection) is a transmembrane tyrosine kinase signaling receptor for members of the GDNF family of ligands. In Chapter 5 RET immunohistochemistry (IHC), double labeling immunofluorescence (IF) and *in situ* hybridization (ISH) was used in adult naïve and nerve injured rats, to study the distribution of RET in the spinal cord. In the dorsal horn, strong RET-immunoreactive (-ir) fibers were abundant in lamina II_{inner} (II_i) and clusters of varicose fibers were found in the deeper layers, although this labeling was preferentially observed after an antigen unmasking procedure. Following dorsal rhizotomy, the fibers in lamina II_i and the varicose fibers completely disappeared from the dorsal horn, indicating that they were all primary afferents. Following peripheral axotomy, RET-ir in primary afferents decreased in lamina II_i and appeared to slightly increase in lamina III and IV. RET-ir was also observed in neurons and dendrites throughout the dorsal horn. Some RET-ir neurons in lamina I had the morphological appearance of nociceptive projection neurons, which was confirmed by the finding that 54% of RET-ir neurons in lamina I colocalized with neurokinin-1. In the ventral horn, RET-immunoreactivity (-ir) was strongly expressed by motoneurons, with the strongest staining in small, presumably gamma-motoneurons, since they did not express cholinergic boutons and m2 muscarinic acetylcholine receptor-ir on their plasma membrane, in contrast to alpha-motoneurons. Increased RET expression following peripheral axotomy was most pronounced in alpha-motoneurons. The expression and regulation pattern of RET in the spinal cord is in line with its involvement in regenerative processes following nerve injury. The presence of RET in dorsal horn neurons, including nociceptive projection neurons, suggests that RET also has a role in signal transduction at the spinal level. This role may include mediating the effects of GDNF released from nociceptive afferent fibers.

In Chapter 6 we have applied the criteria that are generally used to define a neurotransmitter, on the currently available data on GDNF with respect to its involvement in nociceptive transmission. It was concluded that these data are in agreement with a role of GDNF as a neuromodulator in spinal pain transmission, although synthesis in the DRG and biological effects from endogenously released GDNF should be demonstrated to establish such a role unambiguously.

SAMENVATTING

Het doel van dit proefschrift was de betrokkenheid van Glial Cell Line-Derived Neurotrophic Factor (GDNF) bij spinale pijntransmissie te bestuderen. In Hoofdstuk 1 worden enkele recente ontwikkelen betreffende de organisatie van het nociceptieve systeem besproken, gevolgd door een beschrijving van de betrokkenheid van neurotrofe factoren in sensibele transmissie. Dit is de achtergrondinformatie die nodig is om de bevindingen in de volgende hoofdstukken te kunnen interpreteren.

In Hoofdstuk 2 wordt gebruik gemaakt van immunocytochemie om GDNF in het ruggenmerg van de rat aan te tonen. Sterke GDNF labeling werd gevonden in vezels en eindigingen in lamina I, in het buitenste gedeelte van lamina II (IIouter) en in mindere mate in de overige lagen van het ruggenmerg. Enkele spinale ganglion cellen bevatten ook GDNF. Na doorsnijding van de dorsale wortel was GDNF niet meer zichtbaar in de dorsale hoorn van het ruggenmerg en na onderbinding van de dorsale wortel accumuleerde GDNF alleen aan de ganglion zijde van de onderbinding. Deze bevindingen tonen dus anterograad transport van GDNF in primair afferente vezels aan, welke laatstgenoemde vezels de enige bron van GDNF labeling in de dorsale hoorn vormen. De sterke labeling van GDNF in de oppervlakkige dorsale hoorn doet vermoeden dat GDNF een rol speelt bij de pijntransmissie in het ruggenmerg van de volwassen rat.

In Hoofdstuk 3 werd het effect van doorsnijding van de nervus ischiadicus op de expressie van GDNF eiwit onderzocht, met behulp van immunohistochemie. Beeldanalyse van foto's van de ruggenmergcoupes toonde een afname aan van 44% ten opzichte van de contralaterale niet-doorsneden kant na 5 dagen. Dit verschil nam toe tot meer dan 80% na 10 dagen en bleef zo groot tot 100 dagen na de doorsnijding. De snelle en sterke afname suggereert een actieve downregulatie van GDNF eiwit na perifere axotomie. De door ons waargenomen afname van GDNF labeling werd vergeleken met de regulatie van andere peptiden en eiwitten in primaire afferenten en werd bediscusseerd in het kader van een rol van GDNF hetzij als neurotrofe factor of als neurotranmitter.

In Hoofdstuk 4 werd het effect van GDNF en Brain-Derived Neurotrophic Factor (BDNF) injecties door middel van lumbaalpuncties onderzocht, op de expressie van de Immediate Early Gene (IEG) products c-Fos, c-Jun en Krox-24, in de dorsale hoorn van de volwassen rat. In de dorsale hoorn van S1-ruggenmergsegmenten veroorzaakten GDNF en BDNF een sterke toename van het aantal neuronen dat gelabeled was met de verschillende IEGs, welke toename het meest uitgesproken was in lamina I en II (met een factor 2,9-4,5). Meer distaal van de injectieplaats, in de dorsale hoorn van L1/L2 segmenten, was de toename minder uitgesproken, wat een concentratie-afhankelijk effect doet vermoeden. Om de effecten van door middel van een lumbaalpunctie geinjecteerd GDNF te kunnen verklaren, onderzochten we of neuronen in de dorsale hoorn van het lumbo-sacrale ruggenmerg RET tot expressie brachten. RET is het signaal-transducerende element van het receptor-complex voor GDNF. RET immunoreactiviteit bleek aanwezig te zijn in een beduidend aantal neuronen in de dorsale hoorn en sommige RET-immunoreactieve neuronen hadden het morfologische aspect van nociceptief-

specifieke cellen, wat in overeenstemming is met een veronderstelde rol van GDNF in pijntransmissie. De resultaten in dit hoofdstuk tonen aan dat intrathecale GDNF en BDNF IEG expressie in dorsale hoorn neuronen induceren, zeer waarschijnlijk via activatie van hun respectievelijke receptoren die aanwezig zijn op deze neuronen. De suggestie dringt zich daarom op dat endogeen released GDNF en BDNF uit primaire afferenten, welke wordt uitgelokt door nociceptieve stimulatie, betrokken zijn bij de inductie van veranderingen in spinale nociceptieve transmissie die optreden bij verschillende soorten chronische pijn.

RET (REarranged during Transfection) is een transmembraan tyrosine kinase receptor die de signaal transductie voor leden van de GDNF familie verzorgt. In Hoofdstuk 5 wordt de distributie van RET in het ruggenmerg bestudeerd in normale volwassen ratten en ratten met een zenuwbeschadiging, met behulp van immunohistochemie, double-labeling immuunfluorescentie en in situ hybridisatie. In de dorsale hoorn waren sterk gelabelde RET-immunoreactieve (-ir) vezels in ruime mate aanwezig in het binnenste gedeelte van lamina II (IIinner) en clusters van variceuze vezels werden aangetroffen in de diepere lagen. Dit labeling patroon werd voornamelijk gezien na een antigen-unmasking methode. Na doorsnijding van de dorsale wortel, verdween de RET labeling in lamina IIinner en in de variceuze vezels volledig, wat impliceert dat al deze vezels primaire afferenten zijn. Na doorsnijding van de nervus ischiadicus verminderde RET labeling in lamina IIinner en was er een geringe toename in lamina III en IV. RET werd ook waargenomen in neuronen en dendrieten in de gehele dorsale hoorn. De bevinding dat sommige RET neuronen het morfologische aspect van nociceptieve projectieneuronen hadden werd bevestigd door het gegeven dat 54% van de RET-ir neuronen in lamina I ook neurokinin-1 tot expressie brachten. In de ventrale hoorn werd RET-ir sterk tot expressie gebracht in motoneuronen, met de sterkste labeling in kleine, zeer waarschijnlijk gamma-motoneuronen. Dat het gaat om gamma-motoneuronen werd ondersteund door het feit dat deze kleine RET-ir neuronen geen m2 muscarine aceylcholine receptor-ir en cholinerge boutons op hun celmembraan tot expressie brachten, in tegenstelling tot grote alfa-motoneuronen. De toename van RET-ir na doorsnijding van de nervus ischiadicus was het meest uitgesproken in alfa-motoneuronen. Het expressie en regulatie patroon van RET in het ruggenmerg stemt overeen met de bekende betrokkenheid van RET bij regeneratieve processen na zenuwbeschadiging. De aanwezigheid van RET-ir in dorsale hoorn neuronen, waaronder nociceptieve projectie neuronen, suggereert dat RET ook een rol speelt bij signaaltransductie op spinaal niveau, waaronder het mediëren van effecten van GDNF released uit nociceptieve primaire afferente vezels.

In Hoofdstuk 6 zijn de criteria die worden gebruikt om een neurotransmitter te definiëren vergeleken met de data die tot op heden bekend zijn over GDNF voor zover het diens rol in nociceptieve transmissie betreft. Er werd geconcludeerd dat deze data in overeenstemming zijn met een rol van GDNF als neuromodulator in spinale pijntransmissie. Om deze claim ontegenzeggelijk te bewijzen is het echter nodig om synthese van GDNF mRNA in het dorsale ganglion en biologische effecten van endogeen released GDNF aan te tonen.

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Pictorial materials
Joost L.M. Jongen except:

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