NEUROTROPHIC FACTORS IN PERIPHERAL NERVOUS SYSTEM DEVELOPMENT: FUNCTION & SPECIFICITY

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ABSTRACT

Neurotrophic factors control a range of activities in developing neurons, including survival, proliferation, migration and neuronal differentiation. Two classes of neurotrophic factors that are strongly linked to neuronal development of the peripheral nervous system are the neurotrophins and GDNF family ligands. Like other trophic factors they act by triggering signalling through receptor tyrosine kinases. This thesis addresses novel functions for the receptor of GDNF family ligands in peripheral nervous system development and how specificity is achieved at different levels of the trophic factor signalling pathway.

In paper I, NT3, which has been considered to be a promiscuous neurotrophin, is demonstrated to only signal through the TrkC receptor, despite the presence of TrkB. In genetically modified mice, NT3/TrkC, but not NT3/TrkB, could promote survival in the majority of cochleovestibular neurons. Furthermore, NT3/TrkC could not substitute for BDNF/TrkB in final target innervation of the vestibular system of the inner ear.

In paper II and III, Ret, the receptor for GDNF family ligands, is shown to require the binding of the adaptor protein Frs2 to its phosphorylated tyrosine 1062 for migration. In addition, phosphorylated tyrosine 981 was also necessary for a full migratory response, showing cooperation between these tyrosines. Moreover, the subcellular localization of Ret was dependent on which adaptor that binds to tyrosine 1062, highlighting a new function for adaptor proteins.

In paper IV we have ablated Ret in a subpopulation of nociceptive neurons in vivo. We show that Ret was important for the proper expression of ion channels and G protein-coupled receptors. The loss of Ret also led to behavioural changes when mechanical and hot stimuli were applied to the genetically modified mice. This demonstrates the necessity of Ret for normal development of mechano- and thermosensation.

In conclusion this thesis contributes to the elucidation of how specificity in neurotrophic factor signalling is achieved and the definition of new roles for neurotrophic factors in development.
LIST OF PUBLICATIONS

I. Stenqvist A*, Agerman K*, Marmigère F, Minichiello L, Ernfors P.
Genetic evidence for selective neurotrophin 3 signalling through TrkC but not TrkB in vivo.

II. Lundgren TK, Stenqvist A, Scott RP, Pawson T, Ernfors P.
Cell migration by a FRS2-adaptor dependent membrane relocation of Ret receptors.
*J Cell Biochem. 2008 Jan 11; [Epub ahead of print]

Subcellular receptor redistribution and enhanced microspike formation by a Ret receptor preferentially recruiting Dok.
Submitted

Functional implications of Ret deficiency for mechano- and thermosensation.
Submitted

*shared first authorship
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<th>Description</th>
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<tbody>
<tr>
<td>ARTN</td>
<td>Artemin</td>
</tr>
<tr>
<td>ASIC</td>
<td>acid-sensing ion channel</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>Frs2</td>
<td>fibroblast growth factor receptor substrate 2</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>Dok</td>
<td>downstream of kinase</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion/ganglia</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>Gab</td>
<td>Grb2-associated protein</td>
</tr>
<tr>
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<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFL</td>
<td>GDNF family ligand</td>
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<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
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<td>glycosyl-phosphatidylinositol</td>
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<tr>
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<td>growth factor-bound protein</td>
</tr>
<tr>
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<td>isolectin B4</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol tris-phosphate</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>Mrgpr</td>
<td>mas-related G protein-coupled receptor</td>
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<tr>
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<td>nerve growth factor</td>
</tr>
<tr>
<td>NRTN</td>
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</tr>
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</tr>
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<td>phosphatidylinositol-3′-kinase</td>
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<td>phospholipase C gamma</td>
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<td>peripheral nervous system</td>
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<tr>
<td>PSPN</td>
<td>Persephin</td>
</tr>
<tr>
<td>PTB</td>
<td>phospho-tyrosine-binding (domain)</td>
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<tr>
<td>Ret</td>
<td>rearranged during transfection (RTK)</td>
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<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SH</td>
<td>Src homology (domain)</td>
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<tr>
<td>Shc</td>
<td>SH2-containing (adaptor)</td>
</tr>
<tr>
<td>Src</td>
<td>sarcoma gene product (kinase)</td>
</tr>
<tr>
<td>Trk</td>
<td>tropomysin-related kinase (RTK)</td>
</tr>
<tr>
<td>Trp</td>
<td>transient receptor potential (cation channel)</td>
</tr>
<tr>
<td>Y</td>
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1 INTRODUCTION

1.1 NEUROTROPHIC FACTORS AND RECEPTOR TYROSINE KINASE SIGNALLING

1.1.1 Neurotrophic factors

The concept of target-secreted factors for neurons was postulated already during the first half of the 20th century. At the time, Samuel Detwiler and Viktor Hamburger demonstrated that the number of sensory neurons in dorsal root ganglia of amphibian embryos was dependent on the limb they innervate. When they transplanted an additional limb bud, the number of neurons increased, and reversibly the number decreased when a limb bud was removed. However, at this time it was not clear by what mechanism the neuronal numbers were affected. In the 1940s Rita Levi-Montalcini and Viktor Hamburger discovered that neurons die during normal development and that removing a limb bud in chick caused excessive neuronal death. This finding led to the neurotrophic factor hypothesis stating that the target of innervation secretes a limited amount of trophic factor that is taken up by the nerve terminal and supports the survival of the neuron. Today we know that cells that do not receive enough neurotrophic support will die by apoptosis during the period of naturally occurring cell death. In this way the number of neurons is matched with the target size.

Currently, several neurotrophic factors have been identified with diverse effects in the nervous system. Examples of these are neurotrophins, GDNF family ligands, ciliary neurotrophic factor, leukemia inhibitory factor, fibroblast growth factors (FGFs) and insulin-like growth factor (IGF). Recently a new neurotrophic factor, conserved dopamine neurotrophic factor (CDNF), was discovered (Lindholm et al. 2007). Neurotrophic factors are secreted peptides that dimerize and activate receptor tyrosine kinases expressed in neurons and neuronal precursors. In addition to their role in survival, they are involved in early developmental events such as patterning and proliferation and also play important roles during migration, innervation and differentiation of the neuron. Some neurotrophic factors are also important during adulthood for maintenance of the trophic state of the neuron and exert neuroprotective
effects during injury. The majority do also act on other cell types outside of the nervous system.

There are three prevalent modes of action of neurotrophic factors: autocrine, paracrine and target-derived. In autocrine signalling the neurotrophic factor is secreted by the same neuron that also respond to the signal by expressing the corresponding receptor. In paracrine signalling a nearby cell releases the factor that will trigger the response of the neuron. If the factor is target-derived, it implies that the innervation target of the neuron secretes the factor. In this case the neurotrophic factor bound to its receptor can be retrogradely transported through the axon to the soma, where some of the effects are exerted. Likewise neurotrophic factors can be anterogradely transported from the soma to the tip of the axon, where they are released postsynaptically.

Neurotrophins and/or GDNF family ligands have effects on virtually all classes of neurons of the peripheral nervous system (PNS) during development. These neurotrophic factors are the main focus of this thesis and a more detailed description of their properties is given in chapter 1.2 and 1.3.

1.1.2 Neurotrophic factor signalling

1.1.2.1 Receptor tyrosine kinases
Trophic factors signal through receptor tyrosine kinases (RTKs), which appear to be an evolutionary solution to facilitate cell-to-cell communication in multicellular organisms (Hunter and Cooper 1985; King 2004). So far RTKs have only been found in one unicellular organism (King and Carroll 2001). RTKs are transmembrane proteins with an intracellular kinase domain and feature a wide range of different extracellular structures for the purpose of ligand engagement (Pawson 2002). Upon binding of a trophic factor, RTKs dimerize and trans-autophosphorylate the tyrosines in the activation loop of the kinase domain (Schlessinger 2000). This phosphorylation further facilitates the phosphorylation of other intracellular tyrosines on the receptor. Once phosphorylated, these tyrosines make up docking sites for adaptor and effector proteins with phospho-tyrosine binding domains. Adaptor proteins can further recruit other scaffolding and effector proteins to create a multicomponent signalling complex on the receptor. Depending on the constituents of the complex and the availability of other
signalling components, certain intracellular signalling pathways will be triggered. These pathways will ultimately affect transcription, intracellular calcium levels and/or cytoskeletal rearrangements, generating a biological response to the trophic factor signal. Many RTKs also exist in oncogenic forms, for example as the result of chimeric fusions to other proteins or point mutations that render them constitutively active.

1.1.2.2 Domains and modules
Intracellular signalling downstream of RTKs is constructed in a modular fashion (Pawson 2002). These modules can recognize different motives such as modified peptides and lipids or display a catalytic activity. SH2 and PTB domains bind phosphorylated tyrosines, while FHA domains associate with phosphorylated threonines. SH3 domains do not recognize a post-translational modification, but binds to proline-rich motives. PH domains recognize lipids modified by phosphoinositides and in this way can recruit the protein to the membrane. By combining a limited number of functional modules present in the genome, a great number of signalling proteins exist within the cell, including scaffolds, enzymes and transcription factors. Via the different domains, these proteins are able to interact and transmit the signal. The modules are important for the specificity of intracellular signalling, by assuring which signalling proteins are brought into proximity of each other and where in the cell these interactions take place.

Adaptor proteins are a class of scaffolding proteins that dock to activated RTKs by SH2 or PTB domains (Schlessinger and Lemmon 2003). The affinity between a phosphotyrosine and a certain SH2 or PTB domain is determined by the amino acids surrounding the tyrosine. Thus different adaptors bind different phospho-tyrosines. Adaptors lack enzymatic activity, but act by recruiting other scaffolds and effectors. This is facilitated by the phosphorylation of tyrosines on the adaptor itself and the additional domains present within the adaptor.

1.1.2.3 Classical signalling pathways
RTKs activate a number of intracellular signalling pathways or cascades. Originally, they were discovered as simple linear pathways, but today we know that they branch and even interact with each other at multiple levels. Some commonly activated signalling pathways are described below.
The phosphatidylinositol 3 (PI3) kinase is mainly associated with cellular processes such as survival and proliferation. Activated PI3 kinase generates 3-phosphorylated phosphoinositides (i.e. PIP2 and PIP3) that facilitate the activation of other kinases (i.e. PDK-1), and the recruitment of proteins to cellular membranes. One such protein is the serine/threonine kinase Akt. Akt, which is phosphorylated at the membrane by PDK-1, becomes activated and phosphorylates a number of substrates influencing survival. Examples of this are the phosphorylation of Bad and GSK3β that otherwise promote apoptosis. Phosphorylation of IκB by Akt leads to its degradation and release of the transcription factor NFκB that promotes the transcription of pro-survival genes.

The mitogen-activated protein (MAP) kinase signalling cascade is involved both in neuronal survival and differentiation. It is initiated by the activation of a small GTPase (i.e. Ras) via a nucleotide exchange from GDP to GTP, a process facilitated by a guanine nucleotide exchange factor (GEF). The active GTPase binds and activates the serine/threonine kinase Raf. In turn, Raf activates MEK1 and 2 that subsequently phosphorylates ERK1 and 2. Active ERK1/2 translocate to the nucleus where they effect gene expression by activating transcription factors such as Elk1, STATs and Myc.

Phospholipase C gamma (PLCγ) 1 catalyzes the hydrolysis of phosphoinositides to yield diacylglycerol (DAG) and inositol tris-phosphate (IP3). DAG stimulates DAG-regulated protein kinase C (PKC) isoforms, while IP3 promotes release of Ca²⁺ from internal stores. The released Ca²⁺ activates enzymes such as Ca²⁺-regulated PKC isoforms and Ca²⁺-calmodulin-regulated protein kinases. PLCγ signalling is implicated in diverse processes such as survival and neuronal branching.

GEFs make up an interesting category of signalling molecules for cross-talk between different signalling pathways. Apart from being a link in the linear signalling between RTKs and ERK1/2, GEFs can also be activated by DAG, Ca²⁺ and protein kinase A.

1.1.2.4 Specificity in signalling

A central question in the field of signalling addresses how different biological responses are generated by RTKs. For example, the activation of the RTK Ret in enteric neuronal precursors induces migration (Young et al. 2001; Natarajan et al.
2002), whereas activation in nociceptive neurons causes the expression of ion channels and maintenance of the trophic state (Luo et al. 2007). This stresses the importance of the cellular milieu that receives the signal and the competence of the cell. For example, what signalling components and transcription factors are available and what is their subcellular location. In contrast, epidermal growth factor signalling in rat medulloblastoma (PC12) cells leads to proliferation, while nerve growth factor signalling instead induces differentiation (Marshall 1995). This line of evidence suggests that there are intrinsic differences in RTK signalling. It is possible that the RTKs reside in different subcellular locations and are able to recruit different sets of adaptors and/or trigger a different set of signalling pathways. In the study by Marshall et al. it was shown that the resulting length of activation of MAPK was different and that this was crucial for the different biological responses. One could envision that both the cellular milieu and the properties of a trophic factor/RTK pair affect the biological outcome.

1.2 NEUROTROPHINS AND TRK RECEPTORS

1.2.1 A promiscuous system?

1.2.1.1 Neurotrophins and cognate Trk receptors

The neurotrophin family consists of four members in the mouse: Nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), Neurotrophin-3 (NT3) and Neurotrophin-4 (NT4). Neurotrophins are synthesized as precursors (pre-pro-proteins) that can be subsequently proteolytically cleaved to its mature form. The secreted mature form acts as a non-covalently linked homodimer on the Trk RTKs. NGF was the very first neurotrophic factor to be identified. It was isolated in the 1950’s as a nerve growth activity for sensory and sympathetic neurons encountered in snake venom and the mouse submandibular glands (Levi-Montalcini 1987). Thirty years later a second neurotrophin, BDNF, was isolated from pig brain and the corresponding mRNA cloned (Barde et al. 1982; Leibrock et al. 1989). This was in turn followed by several research groups cloning NT3 and NT4 based on sequence homology (Leibrock et al. 1989; Ernfors et al. 1990; Jones and Reichardt 1990; Maisonpierre et al. 1990; Hallbook et al. 1991).
The Trk (tropomysin-related kinase) receptors contain 2 immunoglobulin domains in their extracellular region that are important for neurotrophin binding. In parallel to the discoveries of neurotrophins, TrkA was identified as a chimeric oncoprotein in humans (Martin-Zanca et al. 1986; Martin-Zanca et al. 1989) and TrkB was cloned in mouse (Klein et al. 1989). NGF was then shown by several research groups to bind TrkA (Cordon-Cardo et al. 1991; Hempstead et al. 1991; Kaplan et al. 1991a; Kaplan et al. 1991b; Klein et al. 1991a; Nebreda et al. 1991) and BDNF to bind TrkB (Klein et al. 1991b; Soppet et al. 1991; Squinto et al. 1991). At the same time TrkC was cloned and shown to be a receptor for NT3 (Lamballe et al. 1991). Finally NT4 was found to be a ligand for TrkB (Ip et al. 1992; Klein et al. 1992). (Fig. 1)

1.2.1.2 NT3 infidelity

Some studies in vitro have demonstrated that NT3 is a promiscuous ligand. This includes studies in fibroblasts and neuronal cell lines, showing activation and signalling through the non-cognate receptors TrkA and TrkB (Lamballe et al. 1991; Soppet et al. 1991; Squinto et al. 1991; Ip et al. 1993) (Fig. 1). The fact that some studies have failed to detect this, points at the existence of mechanisms that can modulate NT3 binding preferences, which of some are mentioned below. Using embryonic sensory neurons devoid of TrkC receptors, NT3 has been shown to be able to promote survival through TrkA and TrkB, although at high, possibly non-physiological concentrations (Davies et al. 1995). The in vivo literature, partly summarized below, contains numerous examples from knock-out studies that suggest that NT3 is indeed promiscuous, albeit with very little direct evidence. See also chapter 2.1 for a discussion.

![Figure 1. Neurotrophin and Trk receptor pairs. NGF binds TrkA. BDNF and NT4 share the TrkB receptor. The cognate receptor for NT3 is TrkC, but NT3 can also activate TrkA and TrkB in vitro.](image-url)
1.2.1.3 The p75<sup>NTR</sup> receptor

A fourth receptor that can bind neurotrophins is p75<sup>NTR</sup>. The presence of p75<sup>NTR</sup> has been shown to alter the responsiveness of Trk receptors to neurotrophins. NGF activation of TrkA is potentiated, while NT3 activation of TrkA or TrkB becomes less efficient (Benedetti et al. 1993; Clary and Reichardt 1994; Lee et al. 1994; Mahadeo et al. 1994; Verdi et al. 1994; Bibel et al. 1999). In this way p75<sup>NTR</sup> seems to favour Trk signalling by cognate ligands. Apart from affecting the affinity between neurotrophins and Trk receptors, neurotrophin-activated p75<sup>NTR</sup> can also signal independently of Trk receptors.

1.2.2 Trk receptors in development

1.2.2.1 Expression in the PNS

Trk receptors are expressed both in the CNS and PNS, as well as in non-neuronal tissue. In the PNS this includes neurons of sensory ganglia, the superior cervical ganglion and the enteric nervous system. Correspondingly, neurotrophins are expressed in and around the neurons, along projection paths of nerves and in target tissue.

Transcripts encoding TrkC are detected in early sympathetic development, before the coalescence of the superior cervical ganglion. TrkC expression decreases significantly after E15.5 and remains detectable only in a small subpopulation of cells. TrkA expression on the other hand appears at E13.5, becoming robust from E15.5 onward. Nt3 is synthesized around sympathetic ganglia before E15 (Verdi et al. 1996), but is no longer detected at E15.5. Nt3 is expressed in some but not all targets of the superior cervical ganglion at E17.5, including blood vessels and some glands (Francis et al. 1999).

In the trigeminal ganglion TrkB and TrkC appear at E10.5, while TrkA appears one day later (Huang et al. 1999). At E11.5 there is a limited co-expression that has ceased before E13.5. Accompanying the changing expression of Trk receptors are matching changes in the expression of neurotrophins. The levels of Bdnf and Nt3 are initially highest in the mesenchyme through which the axons grow to the periphery, whereas Ngf is expressed predominantly in the target field epithelium (Davies et al. 1987; Arumae et al. 1993; Buchman and Davies 1993). Later in development, Nt3 is largely...
confined to the oral epithelium and the mesenchyme underlying the skin (Wilkinson et al. 1996). For a description of dorsal root ganglia and the cochleovestibular system see 1.4.2 and 1.4.3.

1.2.2.2 Findings from knock-out mice

TrkA \(^{−/−}\) and Ngf \(^{−/−}\) mice appear normal at birth but fail to thrive and instead die during an extended period postnatally (Crowley et al. 1994; Smeyne et al. 1994). They show an almost complete loss of neurons in the superior cervical ganglion, which is caused by a neuronal death that starts before birth and continues postnatally. In the trigeminal ganglion about 75% of the neurons are lost in both knock-out strains.

TrkB \(^{−/−}\) and Bdnf \(^{−/−}\) mice appear normal at birth, but most die within the three first postnatal weeks (Klein et al. 1993; Ernfors et al. 1994a; Jones et al. 1994; Conover et al. 1995). In contrast, Nt4 \(^{−/−}\) mice are viable and fertile (Conover et al. 1995; Liu et al. 1995). The neuronal death in sensory ganglia has been described as either similar between TrkB \(^{−/−}\) and Bdnf \(^{−/−}\) mice or enhanced in the TrkB \(^{−/−}\) animals. In the nodose-petrosal ganglion for example, the neuronal loss in TrkB \(^{−/−}\) mice is 95%, while it is only 57% in the Bdnf \(^{−/−}\) mice (Liu et al. 1995; Silos-Santiago et al. 1997). Complementary to this, Nt4 \(^{−/−}\) mice show a 59% and double mutant Nt4 \(^{−/−};\) Bdnf \(^{−/−}\) mice a 90% reduction in the same ganglion, highlighting the dependence on separate neurotrophins for survival of different TrkB expressing subpopulations of the nodose-petrosal ganglion (Liu et al. 1995).

TrkC \(^{−/−}\) and Nt3 \(^{−/−}\) mice have similar movement deficits and die shortly after birth (Ernfors et al. 1994b; Klein et al. 1994). A recurring theme when comparing TrkC \(^{−/−}\) and Nt3 \(^{−/−}\) mice is that the neuronal death is commonly greater in the Nt3 \(^{−/−}\) animals. (Ernfors et al. 1994b; Farinas et al. 1994; Tessarollo et al. 1997). The biggest difference is observed in the sympathetic superior cervical ganglion where Nt3 \(^{−/−}\) mice display approximately a 50% neuronal loss, whereas the TrkC \(^{−/−}\) mice have normal neuronal numbers. Other ganglia such as the trigeminal, nodose-petrosal and geniculate display two- to threefold difference in neuronal loss. It has been proposed that these differences are due to a more general dependence on NT3. This dependence could be mediated at early time points through more widely expressed TrkC or through promiscuous interactions with TrkA and TrkB. For phenotypes in dorsal root, cochlear and vestibular ganglia see chapter 1.4.2 and 1.4.3.
1.2.2.3 Trk receptor splicing

Trk receptors are alternatively spliced, leading to altered susceptibility to neurotrophin signalling. Differential splicing of the extracellular domain of all three receptors affect ligand interactions (Clary and Reichardt 1994; Strohmaier et al. 1996). Truncated forms of TrkB and TrkC exist that lack the tyrosine kinase domain (Valenzuela et al. 1993). The function of these isoforms in non-neuronal cells could include presentation of the neurotrophin to the neuron or sequestration of the ligand. Within the neuron, these receptors could inhibit receptor dimerization between full-length receptors, thus interfering with intracellular signalling. However, in knock-out mice where only the kinase domain has been removed, some kinase-independent pro-survival signalling seems to persist (Minichiello et al. 1995) as compared to complete knock-outs (Tessarollo et al. 1997). Furthermore, intracellular signalling is altered downstream of Trk receptors with an amino acid insert within the tyrosine kinase domain (Tsoulfas et al. 1996; Meakin et al. 1997).

1.2.3 Trk receptors and intracellular signalling

1.2.3.1 Tyrosines, adaptors and signalling pathways

Trk receptors contain ten evolutionarily conserved intracellular tyrosines that can be phosphorylated. Three of these, Y670, Y674 and Y675 (human TrkA sequence nomenclature) are present in the autoregulatory loop of the kinase domain. Apart from being important for activation of the kinase, they do also bind adaptor proteins such as Grb2, rAPS and SH2B (Qian et al. 1998; MacDonald et al. 2000). The most extensively studied tyrosines are Y490 and Y785. Y785 has been shown to recruit PLCγ1 and the kinase CHK (Obermeier et al. 1993; Yamashita et al. 1999). PLCγ1 activation by NGF stimulates PKCδ, which leads to the activation of ERK1/2 and neurite outgrowth in PC12 cells (Corbit et al. 1999). Y490 interacts with the adaptor proteins Shc and Frs2 (Stephens et al. 1994; Meakin et al. 1999). Frs2 can recruit Crk which leads to the activation of the GEF C3G. C3G activates the GTPase Rap1 that stimulates B-Raf, resulting in the prolonged activation of ERK1/2 (York et al. 1998; Meakin et al. 1999). Shc engagement of TrkA via Y490 can activate two different pathways through the recruitment of Grb2. Grb2 bound to the GEF SOS will activate Ras and subsequently via Raf trigger transient ERK1/2 activity (Stephens et al. 1994). Alternatively, Grb2 associates with Gab1 and in this way activates Akt via PI3 kinase (Holgado-Madruga et
An *in vivo* silencing tyrosine to phenylalanine mutation of Y490 in *TrkB* or *TrkC* leads to comprised phosphorylation of ERK1/2 and almost abolished activation of Akt, showing the relative contributions of this tyrosine to the two signalling pathways. In these mice the phenotype is milder regarding neuronal death in the inner ear than in mice completely devoid of the receptor, implicating cooperation between phosphorylated tyrosines for survival (Minichiello et al. 1998; Postigo et al. 2002). For a summary see figure 2.

**Figure 2.** A selection of signalling pathways that can be activated downstream of Trk receptors.

### 1.2.3.2 Internalization

After activation, the NGF/TrkA complex can be internalized. This compartmentalizes the signalling complex and facilitates its transport to other parts of the cell, which might regulate the signalling cascades activated. When NGF binds to TrkA, local downstream signalling can for example affect growth cone turning or exocytosis. In contrast other responses require changes in gene transcription whereby the signal must be transduced to the nucleus. Endosomes containing NGF/TrkA have been shown be retrogradely...
transported from the axon terminal to the cell soma (Grimes et al. 1996; Bhattacharyya et al. 1997; Riccio et al. 1997), and it has been demonstrated in sensory neurons that while neurotrophins stimulate local activation of ERK1/2 and ERK5 in axons and cell bodies, only ERK5 is activated during retrograde signalling (Watson et al. 2001).

1.2.3.3 Cross-talk
RTKs are not only activated by their proper ligands, but also can be transactivated by other receptors. TrkA has been demonstrated both to be activated by G protein-coupled receptors (GPCRs) and to be able to transactivate other RTKs by slow kinetics. In PC12 cells the activation of GPCRs, by adenosine or PACAP, leads to the transactivation of TrkA and seems to involve Src kinases (Lee and Chao 2001; Lee et al. 2002). The TrkA receptor also has the capacity to activate the long isoform of Ret in postnatal superior cervical ganglion neurons (Tsui-Pierchala et al. 2002b). The presence of Ret is necessary to observe full trophic response to NGF in these cells.

1.3 GDNF FAMILY LIGANDS AND RET

1.3.1 A three component system

The GDNF family of neurotrophic factors includes four members: Glial cell line-derived neurotrophic factor (GDNF), Neurturin (NRTN), Artemin (ARTN) and Persephin (PSPN). The mature GDNF family ligands (GFLs) are biologically active as covalently bound homodimers. They are synthesized as precursors (pre-pro-proteins) with an amino-terminal signal sequence that is cleaved upon secretion and a prosequence that is cleaved from the mature polypeptide (Lin et al. 1993). The first member of this family to be discovered was GDNF. It was originally extracted from a rat glial cell line based on its potent survival effect on embryonic midbrain dopaminergic neurons (Lin et al. 1993). NRTN was found in a Chinese hamster ovary cell line as a survival factor for cultured sympathetic neurons (Kotzbauer et al. 1996). 10 years ago PSPN was cloned by using degenerate PCR (Milbrandt et al. 1998) and ARTN was found using database searches based on homology to NRTN (Baloh et al. 1998b). All GFLs except, PSPN, have been shown to support the survival of diverse populations of PNS neurons in culture (Buj-Bello et al. 1995; Kotzbauer et al. 1996; Baloh et al. 1998b; Milbrandt et al. 1998).
Ret (rearranged during transfection) is the common RTK for the GFLs. The Ret extracellular ligand-binding domain contains four cadherin-like repeats and a Ca\(^{2+}\) binding site (Anders et al. 2001). Ret was initially discovered as a proto-oncogene, activated by DNA rearrangement following transfection of fibroblast cells with DNA from human T-cell lymphoma (Takahashi et al. 1985). In 1996 GDNF was shown to be a ligand for the orphan Ret receptor (Durbee et al. 1996a; Trupp et al. 1996; Vega et al. 1996; Worby et al. 1996).

Many growth factors bind directly to their RTK, but GFLs must first engage with a co-receptor from the GDNF family receptor alpha (GFR\(\alpha\)) family (Fig. 3). The GFR\(\alpha\) family consists of four members that each binds a preferred GFL. GFR\(\alpha\)1 recruits GDNF (Jing et al. 1996; Treanor et al. 1996), GFR\(\alpha\)2 binds NRTN (Baloh et al. 1997; Buj-Bello et al. 1997; Klein et al. 1997; Sanicola et al. 1997), GFR\(\alpha\)3 interacts with ARTN (Baloh et al. 1998a; Naveilhan et al. 1998; Widenfalk et al. 1998) and GFR\(\alpha\)4 binds PSPN (Enokido et al. 1998; Lindahl et al. 2001). At least in vitro, NRTN and ARTN bind weakly to GFR\(\alpha\)1 (Jing et al. 1996; Baloh et al. 1997; Creedon et al. 1997; Baloh et al. 1998b) and GDNF to GFR\(\alpha\)2 (Sanicola et al. 1997), but the physiological significance of these interactions is unclear. GFR\(\alpha\) receptors are tethered to the plasma membrane by a carboxy-terminal glycosyl-phosphatidylinositol (GPI) anchor, but can also be cleaved and act in a soluble form (Paratcha et al. 2001).

**Figure 3.** The receptor tyrosine kinase Ret can be activated by four different ligands; GDNF, Neurturin (NTRN), Artemin (ARTN) and Persephin (PSPN). GFR\(\alpha\) co-receptors facilitate the activation of Ret by binding to their preferred ligand(s).
1.3.2 Ret in development

1.3.2.1 Expression in the PNS

Ret is expressed in many different tissues during development, usually together with one or more GFRα receptors. However, GFRα receptors are more widely expressed than Ret, especially in the CNS (Nosrat et al. 1997; Trupp et al. 1997). GDNF has been shown to trigger Ret-independent signalling through GFRα1 alone, and this may in part explain the biological function of the broad expression of GFRα receptors (Trupp et al. 1999). Outside of the nervous system Ret is present from early on in the developing metanephric kidney (Pachnis et al. 1993). In the PNS, Ret is expressed in the developing autonomic nervous system, the enteric nervous system and the sensory ganglia, suggesting that the Ret signal transduction pathway is involved in development of all major branches of the PNS.

Ret is detected in the majority of migrating enteric precursors emanating from the vagal neural crest, as well as in the postmitotic neurons of the enteric nervous system (Pachnis et al. 1993; Tsuzuki et al. 1995; Durbec et al. 1996b). Initially Ret only co-localizes with GFRα1, but subsequently GFRα2 is also expressed in the enteric nervous system (Nosrat 1997, Golden 1999). Postnatally, Gfrα1 expression weakens, but Gfrα2 persists in the adult. Complementary to this, Gdnf and Ntrn are expressed in surrounding muscle layers of the gut from E10 and E12, respectively (Nosrat et al. 1997; Golden et al. 1999; Young et al. 2001; Natarajan et al. 2002). In the adult Gdnf is not detected.

In sympathetic neuronal precursors Ret is expressed from E9.5 (Pachnis et al. 1993). At E12.5 Gfra1-3 are also abundantly expressed (Nishino et al. 1999). Gfra1 is progressively down-regulated to very low levels postnatally, while Gfra2 and Gfra3 persist, but in a more restricted pattern. Artn can be found en route of sympathetic precursor migration and in arteries along which sympathetic axons project (Honma et al. 2002). Gdnf and Ntrn are expressed in many target tissues of sympathetic innervation (Trupp et al. 1995; Golden et al. 1999).

In parasympathetic precursors Ret and Gfra1 are expressed, but Gfra1 is down-regulated before birth in many parasympathetic cranial ganglia (Enomoto et al. 2000). Gfra2 starts being expressed during embryonic development and persists into
adulthood (Rossi et al. 2000). \textit{Gdnf} is expressed within and around migrating parasympathetic precursors and parasympathetic target tissue, but the mRNA decreases as development proceeds (Golden et al. 1999; Enomoto et al. 2000). \textit{Nrtn} is detected from E14 in target tissue, such as the salivary glands, and is up-regulated postnatally (Golden et al. 1999; Enomoto et al. 2000).

1.3.2.2 Findings from knock-out mice

Studies using knock-out mice for Ret, GFR\(\alpha\) receptors or the GFLs have proven their functional importance during development. The severest phenotypes have been observed in PNS and kidney development and in general the results have strengthened the link between a certain GFL and its preferred GFR\(\alpha\) receptor. Ret\(^{-}\), Gdnf\(^{-}\) and Gfra1\(^{-}\) mice die soon after birth (Schuchardt et al. 1994; Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996; Cacalano et al. 1998; Enomoto et al. 1998). The most striking phenotypes of these mice are the absent or very rudimentary kidneys and the complete lack of enteric neurons in the myenteric plexus of the small and large intestine. In the sympathetic nervous system different phenotypes have been observed. Ret-deficient mice display impaired migration and axonal growth (Durbec et al. 1996b; Enomoto et al. 2001), while GDNF-deficient mice have a 30\% reduction of neurons in the superior cervical ganglion (Moore et al. 1996) and GFR\(\alpha\)1-deficiency does not affect the number of sympathetic neurons at all (Cacalano et al. 1998; Enomoto et al. 1998). In the trigeminal ganglion no deficiencies have been detected at late embryonic or early postnatal stages of Gdnf\(^{-}\) and Gfra1\(^{-}\) mice (Moore et al. 1996; Cacalano et al. 1998; Enomoto et al. 1998). However, in the petrosal ganglion the number of sensory neurons is reduced by 40\% and 15\% in Gdnf\(^{-}\) and Gfra1\(^{-}\) mice, respectively (Moore et al. 1996; Cacalano et al. 1998).

\textit{Nrtn}\(^{-}\) and Gfra2\(^{-}\) mice are viable and fertile, but show various defects in the cholinergic division of the parasympathetic and enteric nervous system (Heuckeroth et al. 1999; Rossi et al. 1999). In the submandibular ganglion parasympathetic neurons are lost, while in the otic and sphenopalantine ganglia the parasympathetic neurons are only reduced in size (Rossi et al. 2000). The defects in dorsal root ganglia are discussed further in chapter 1.4.3.

\textit{Artn}\(^{-}\) and Gfra3\(^{-}\) mice are viable and fertile, but they display migration and axonal projection deficits in the entire sympathetic nervous system (Nishino et al. 1999;
Honma et al. 2002). No sensory neuron deficits have been found in Artn\(^{-/-}\) and Gfra3\(^{-/-}\) mice, although GFR\(\alpha\)3 is expressed in dorsal root ganglia and the trigeminal ganglion.

Pspn\(^{-/-}\) and Gfra4\(^{-/-}\) mice are viable and fertile (Tomac et al. 2002; Lindfors et al. 2006a). Since Ret only co-localizes with GFR\(\alpha\)4 in thyroid C-cells of young individuals (Lindfors et al. 2006a), no PNS defects have been observed in these animals.

1.3.2.3 Ret splicing

Ret is alternatively spliced into three different transcripts (Tahira et al. 1990; Myers et al. 1995). Two of these, Ret9 and Ret51 are found in mouse during development and adulthood (Lee et al. 2003). The resulting isoforms diverge in the intracellular carboxy-terminal and the longer isoform Ret51 contains two additional intracellular tyrosines that can be phosphorylated. Ret9 and Ret51 are expressed in overlapping populations of cells but have not yet been demonstrated to heterodimerize (Tsui-Pierchala et al. 2002a; Scott et al. 2005). Knock-in mice expressing only Ret9 or Ret51 have been generated (de Graaff et al. 2001). The Ret9 mice appeared normal with no overt phenotypic changes, while the Ret51 mice resembled the Ret knock-out, lacking kidneys and parts of the enteric nervous system. However, in a more recent study other knock-in mice expressing only one Ret isoform showed that Ret51 is sufficient to drive development of the kidney and enteric nervous system (Jain et al. 2006). In addition, the latter study showed that signalling downstream of the two isoforms is different.

1.3.3 Ret and intracellular signalling

1.3.3.1 Tyrosines, adaptors and signalling pathways

The short isoform Ret9 carries at least eight intracellular tyrosines that are phosphorylated upon ligand binding of the receptor. Four of these tyrosines (Y752, Y826, Y905 and Y928) reside in the kinase domain. Of those Y905 has been shown to recruit adaptor proteins Grb7 and Grb10 (Pandey et al. 1995; Pandey et al. 1996), and Y752 and Y826 can directly bind the transcription factor STAT3 (Schuringa et al. 2001). Outside of the kinase domain PLC\(\gamma\) docks to Y1015 while Src mainly binds to Y981 (Borrello et al. 1996; Encinas et al. 2004). Src kinases have been shown to be activated by GDNF-stimulation promoting survival and neurite out-growth (Tansey et
Y1062 makes up a multi-docking site for Shc, Frs2, IRS and Dok adaptor proteins (Asai et al. 1996; Grimm et al. 2001; Kurokawa et al. 2001; Melillo et al. 2001a; Melillo et al. 2001b). A silencing mutation of this tyrosine (Y1062F) in knock-in animals has a phenotype very similar to the Ret knock-out and demonstrates the importance of this docking site for signalling during development (Jijiwa et al. 2004; Jain et al. 2006). Y1062 has been demonstrated in vitro to be involved in many of the signalling pathways activated downstream of Ret, including activation of Akt, ERK, Jnk and p38MAPK signalling (Hayashi et al. 2000). Shc at Y1062 has been shown to recruit Grb2 that further can associate with Gab1/2 (Besset et al. 2000; Hayashi et al. 2000). This then leads to the activation of the PI3K/Akt signalling that in turn activates NFκB. Alternatively, Grb2 binds to the GEF SOS which subsequently triggers Ras/ERK signalling, leading to the activation of the transcription factor CREB. Frs2 binding to Y1062 can also recruit Grb2 and SOS and

**Figure 4.** Ret9 and downstream signalling pathways. Active Ret can recruit a number of adaptor and effector proteins. Four adaptors, Shc, Frs2, IRS and Dok, compete for binding to tyrosine 1062.
trigger the Ras/ERK pathway (Kurokawa et al. 2001; Melillo et al. 2001b). Dok proteins interacting with Y1062 have been implicated in neurite out-growth, possibly through ERK1/2 activation (Grimm et al. 2001; Crowder et al. 2004; Uchida et al. 2006). For a summary see figure 4.

Isoform Ret51 has two extra phosphorylated tyrosines. Y1096 can recruit Grb2 and contribute to PI3K and MAPK signalling (Alberti et al. 1998; Besset et al. 2000; Hayashi et al. 2000), thus having a partially redundant function with Y1062 (Degl'Innocenti et al. 2004; Jain et al. 2006). In addition Ret51 have different amino acids just C-terminal to Y1062, changing the binding motif for proteins with SH2 or PTB domains. This seems to lead to differences in the interaction with Shc, but not Frs2 (Kurokawa et al. 2001). Together this shows that GFL signalling can be modified depending on the Ret isoform expressed.

1.3.3.2 Lipid rafts
Lipid rafts are considered cholesterol and sphingolipid enriched microdomains of cellular membranes, which certain proteins have a higher affinity for. These proteins are commonly palmitoylated or GPI-linked. In this way the lipid rafts can function as signalling platforms controlling which proteins that have access to each other. Studies of lipid rafts are based on biochemical membrane fractionations; however, the rafts have not been visualized in living cells (Munro 2003). Inactive Ret resides outside of lipid rafts, but can after GDNF-stimulation be recruited inside by GFRα1 (Tansey et al. 2000; Paratcha et al. 2001). This recruitment leads to differences in downstream signalling and Ret engaging with Shc outside lipid rafts and Frs2 and Src inside (Tansey et al. 2000; Encinas et al. 2001; Paratcha et al. 2001).

1.3.3.3 Cis or trans
Another aspect of Ret signalling is the GFRα engagement either in cis or trans. In neurons Ret is commonly co-expressed with at least one GFRα receptor, thus facilitating cis signalling. However, GFRα receptors can also be expressed by non-neuronal cells, making signalling in trans possible. Soluble GFRα1 has been shown to be released by Schwann cells and innervation targets for sensory neurons, such as the carotid body, and can make up a source of trans-activating GFRα1 (Paratcha et al. 2001; Ledda et al. 2002). Despite this, mice lacking GFRα1 expression in non-Ret expressing cells, do not display a phenotype (Enomoto et al. 2004).
1.4 NEUROTROPHIC FACTORS AND DEVELOPMENT OF SENSORY SYSTEMS

1.4.1 The sensory nervous system

The neurons of the PNS are organized into clusters in the periphery referred to as ganglia. The sensory branch of the PNS includes the dorsal root ganglia (DRG) and the ganglia of the cranial nerves. The neurons of these ganglia are bipolar and receive sensory input from our internal and external environment that is then transmitted to the CNS. A vast array of stimuli can be perceived by the sensory nervous system including temperature changes, pressure, pH and various chemical substances. These stimuli can then for example be interpreted by the CNS as heat, sounds, acidity and pain, respectively; depending on the identity of the sensory neuron and where in the CNS the information is received and processed. Often all neurons of a particular sensory ganglion convey the same modality of sensory information. However, in the DRG and trigeminal ganglion different neurons transmit different modalities. In these ganglia some neurons are even polymodal, responding to several categories of stimuli.

Sensory neurons originate from progenitors that migrate either from the neurogenic placodes or the neural crest to their final location (LeDouarin and Kalcheim 1999). The neural crest is a ridge of cells found at the boundary between the ectoderm and the neural plate, while placodes are thickenings in the ectoderm. Commonly, there is either a placodal or neural crest origin of a certain ganglion, but the trigeminal ganglion contains neurons of both origins. The data below concerns development of the mouse, unless otherwise stated.

1.4.2 The cochleovestibular system

1.4.2.1 Function and organization

The inner ear registers and transduces information regarding hearing (intensity and spectral properties of sound) and balance (position, velocity and acceleration) to the CNS. The cellular bases for this are mechanosensory hair cells residing in sensory epithelial structures that respond to local mechanical perturbations by creating a synaptic potential. This information is then relayed through afferent nerve fibres
belonging to neurons in the cochlear or vestibular ganglia to the cochlear and vestibular nuclei in the brainstem. The brain can in turn modulate the hair cell response and the information sent through afferents by sending efferent projections back to the sensory epithelium.

The cochlear system is made up of the cochlear ganglion that innervates hair cells located in the organ of Corti in the cochlea (Fig. 5A). These hair cells are organized in one row of inner hair cells and three rows of outer hair cells (Fig. 5B). When sound waves causes vibrations in the organ of Corti, the stereocilia, which protrudes from the hair cells, will be pressed against the tectorial membrane causing polarization. In the cochlear ganglion two kinds of neurons are found, type I, which are by far the most abundant (>90%), and type II. One type I neuron innervates one inner hair cell, while one type II neuron can innervate several outer hair cells.

![Figure 5](image)

**Figure 5.** Schematic drawings of the inner ear. A) Overview of the cochleovestibular system. B) The organ of Corti with one row of outer and three rows of inner hair cells. C) The sensory epithelium of a macula with type I and type II hair cells.

The vestibular system is made up of the vestibular ganglion that innervates hair cells in different sensory epithelia of the vestibular organ (Fig. 5A). The vestibule consists of two chambers, the utricle and the saccule, and the three semicircular canals. Theses structures contain sensory epithelia referred to as the utricular and saccular maculae,
and ampullary cristae, respectively. In the maculae two kinds of hair cells exist, the flask shaped type I and the cylindrical type II. The stereocilia of these hair cells press against the overlaying otolithic membrane when the head accelerates or changes position and this causes a polarization of the hair cells. (Fig. 5C). Type I hair cells are surrounded by calyx shaped afferent innervation originating from one vestibular neuron, while type II hair cells are contacted at the base by several afferent nerve fibres.

1.4.2.2  *A placodal origin*

In the adult, the inner ear consists of two different systems for hearing and balance; however, they do have a common developmental origin. All structures and tissues, neuronal, sensory and non-sensory, of the cochleovestibular system are derived from the otic placode. The otic placode is a patch of ectoderm adjacent to the developing hindbrain. The morphological induction of the inner ear starts at embryonic day (E) 8.5, when the otic placode thickens and invaginates, forming the otic cup. At E9.5 the cup closes giving rise to the otocyst. From the dorsolateral wall of the otocyst forms two outpouches that will subsequently give rise to the semicircular canals. Likewise, the ventromedial outpouch that forms gives rise to the cochlea. Exactly when and what induces the formation of the inner ear is not clear, but FGF3 and 10, secreted from the presumptive hindbrain and underlying mesoderm, respectively, have been implicated (Wright and Mansour 2003). These factors are also thought to later be involved in the emergence and formation of the cochlea and vestibule (Mansour et al. 1993; McKay et al. 1996; Pauley et al. 2003). The otic placode can be identified molecularly already at E8 by the expression of the transcription factors Pax8 and Dlx5 (Depew et al. 1999). The patterning of the inner ear leads to the formation of compartments in the otocyst that are marked by the expression of different sets of transcription factors. In some cases inactivation of these genes leads to striking morphologic effects. Examples of this are the removal of Pax2 or Dlx5, which are expressed in the ventromedial and dorsolateral part of the otocyst, respectively. This leads to the loss of the cochlea and malformation of the semicircular canals, respectively (Torres et al. 1996; Acampora et al. 1999).

1.4.2.3  *Cochleovestibular neurogenesis*

How the early expression patterns of transcription factors relates to cell fate of the cochleovestibular neurons is not well understood, but there is data available on the expression of different markers. The proneural gene *neurogenin-1* is initially expressed
in the otic placode and the expression persists through the otic cup and otocyst stages (Ma et al. 2000). The neuronal precursors, which reside in the anteroventral part of the otocyst epithelium, upregulate the proneural genes *neuroD* and *neuroM* before emigration. Delamination of otic neuronal precursors then starts at the otic cup stage and ends by the time of the late otocyst (Carney and Silver 1983). This delamination is impaired in *neurogenin-1* and *NeuroD* null mice (Ma et al. 1998; Liu et al. 2000; Kim et al. 2001). The expression of transcription factor Islet-1/2 is induced in delaminating and migrating cells that also start expressing βIII-tubulin (Memberg and Hall 1995; Camarero et al. 2003), indicating specification of neuronal identity.

The migrating neuronal precursors are proliferative and depend at least in chicken on IGF1 for survival and proliferation (Camarero et al. 2003). Null mutants for FGF3 or FGFR2b show reduced size of the cochleovestibular ganglion, implicating FGF signalling in survival of the neuronal precursors (Pirvola et al. 2000). Since both FGF3 and 10 are expressed in the neurogenic region of the otocyst, but FGFR2b is expressed in neighbouring tissue (Pirvola et al. 2000), this indicates a second FGFR being expressed and activated in the neurogenic region or a reciprocal signal stemming from activated FGFR2b in the adjacent region. After migration the neuronal precursors aggregate, forming the cochleovestibular ganglion that later separate into the cochlear and vestibular ganglia.

1.4.2.4 Final target innervation, neuronal survival and neurotrophins

During target innervation of cochleovestibular sensory epithelia, days after the afferent nerve fibres have first extended, the afferents become attracted by and the neurons dependent on BDNF and NT3 for survival. BDNF and NT3 are already detected at E10 in the sensory patches of the otocyst and show a differential expression with *Nt3* being expressed in the ventral half and *Bdnf* in both ventral and dorsal halves of the otocyst. Later in development this spatial segregation becomes more distinct. At E13, *Nt3* is strongly expressed in the greater epithelial ridge that gives rise to the organ of Corti and in maturing utricular and saccular maculae. At the same time, *Bdnf* can be found in all sensory epithelia, including the ampullary cristae, but with a delayed expression in the basal turn of the cochlea. During embryonic development, *Bdnf* expression is restricted to hair cells of the sensory epithelia, whereas *Nt3* is also expressed by supporting cells (Wheeler et al. 1994). Postnatally, *Bdnf* expression is turned off in the organ of Corti and the *Nt3* expression is limited to the inner hair cells. This coincides with the
reorganization of afferent innervation mainly to the inner hair cells. In the vestibular organ, *Nt3* expression decreases postnatally and is almost completely gone by postnatal day (P) 9, while *Bdnf* continues to be expressed in the adult. (Ernfors et al. 1992; Pirvola et al. 1992; Ylikoski et al. 1993; Farinas et al. 2001).

TrkB and TrkC receptors are co-expressed in cochlear and vestibular neurons during development (Ylikoski et al. 1993; Pirvola et al. 1994; Farinas et al. 2001). Migrating neuronal precursors do not express *TrkB* or *TrkC* (Pirvola et al. 1994; Schecterson and Bothwell 1994; Farinas et al. 2001), but the full-length isoforms can be detected at early stages of cochleovestibular ganglion compaction, when neuronal differentiation starts (Ernfors et al. 1992; Pirvola et al. 1994).

The functional importance of neurotrophin and Trk receptor expression in the cochleovestibular system for target innervation and neuronal survival has been extensively studied in genetically modified mice. In these studies it has become clear that cochlear neurons are strongly dependent on NT3/TrkC signalling, whereas vestibular neurons depend more on BDNF/TrkB signalling. In the postnatal vestibular ganglion about 80% of the neurons are lost in the *TrkB*−/− and *Bdnf*−/− mice and the innervation of the utricular and saccular maculae and ampullary cristae is almost absent (Ernfors et al. 1995; Bianchi et al. 1996; Silos-Santiago et al. 1997). In contrast, only around 30% less neurons are seen in the *TrkC*−/− and *Nt3*−/− mice and this reduction is not accompanied by obvious loss of innervation (Ernfors et al. 1995; Silos-Santiago et al. 1997). In the cochlear ganglion of *TrkB*−/− and *Bdnf*−/− mice 20% and 7%, respectively of the neurons are lost (Ernfors et al. 1995; Silos-Santiago et al. 1997), while in the *TrkC*−/− and *Nt3*−/− mice as much as a 70% and 85% loss is seen, respectively (Ernfors et al. 1995; Silos-Santiago et al. 1997; Tessarollo et al. 1997; Farinas et al. 2001). The first analyses of mutant mice revealed that BDNF- and TrkB-deficient mice are missing nearly all type II neurons that innervate outer hair cells, while NT3-deficient mice instead lack type I neurons that innervate inner hair cells (Farinas et al. 1994; Ernfors et al. 1995; Silos-Santiago et al. 1997). Subsequent studies failed to detect such difference between NT3/TrkC and BDNF/TrkB signalling, but rather demonstrated a differential loss of neurons in the base and apex of the cochlea, respectively (Bianchi et al. 1996; Fritsch et al. 1997; Farinas et al. 2001). This later data can be explained by the spatiotemporal gradient of *Nt3* and *Bdnf* expression in the organ of Corti. Not surprisingly all neurons in both ganglia are lost in the *TrkB*+/−;*TrkC*+/−;*Bdnf*+/−;*Nt3*+/−
double mutant mice (Ernfors et al. 1995; Silos-Santiago et al. 1997). The neuronal
death has been shown to be apoptotic and to take place between E13.5 and E15.5
(Schimmang et al. 1995; Bianchi et al. 1996; Farinas et al. 2001).

Neurotrophins are thought to show a degree of redundancy. To address this issue in the
cochleovestibular system, knock-in mice carrying *Nt3* in place of *Bdnf*, and vice versa,
has been generated (Coppola et al. 2001; Agerman et al. 2003). In both these mice the
gene replacement strategy results in almost normal cochlear neuronal numbers and
target innervation. This demonstrates that both these neurotrophins can to a similar
extent promote survival and target innervation in the cochlea when expressed at the
right time and place. However, in the vestibular system the effects are different. In
animals with *Nt3* expressed from the *Bdnf* locus only half of the neurons remain and
very sparse afferent innervation is seen in the sensory epithelia (Agerman et al. 2003).
This suggests a qualitative difference between NT3 and BDNF signalling.

Effects of neurotrophin signalling on late development of cochleovestibular neurons
have not been investigated in genetic models. Neurotrophins could be involved in the
postnatal remodelling of innervation and the expression of functional markers in the
inner ear, as has been shown for the DRG.

1.4.2.5  *A role for GDNF?*

*Gdnf* starts to be expressed in the cochlear hair cells of guinea pig during the first
postnatal week. After the second postnatal week, this expression is restricted to the
inner hair cells where it persists into adulthood (Ylikoski et al. 1998). Similarly,
GFRα1 is expressed in cochlear neurons of the guinea pig (Ylikoski et al. 1998).
However, Ret does not seem to be expressed in cochlear and vestibular ganglia during
postnatal rodent development (Nosrat et al. 1997). This suggests that GDNF signalling
through GFRα1 and perhaps Ret plays a role during postnatal remodeling of cochlear
innervation and synaptogenesis. GDNF could possibly also influence the final
functional maturation of cochlear neurons by changing their expression of functional
markers, as has been shown for DRG neurons (Luo et al. 2007).
1.4.3 Dorsal root ganglia

1.4.3.1 Function and organization

The DRG are organized bilaterally in pairs along the spinal cord. At different levels of the spinal cord DRG project to different body parts and contain different numbers and composition of neurons. To identify the different DRG, they are named after the corresponding level of the spinal cord (cervical, thoracic, lumbar, and sacral). They project centrally to different layers in the spinal cord and peripherally to diverse end organs depending on the identity of the sensory neuron (Fig. 6). DRG neurons can generally be divided into three main groups: proprioceptive, nociceptive and mechanoreceptive. Large proprioceptive neurons project to muscle spindles and tendon organs, from where they convey information about position and movements of limbs to

![Diagram](image-url)

**Figure 6.** Unilateral view of the spinal cord and a dorsal root ganglion. In dorsal root ganglia three major classes of neurons reside; proprioceptors, mechanoreceptors and nociceptors. They express TrkA, TrkB, TrkC and Ret as indicated in the figure. Different classes of neurons project to different lamina of the spinal cord. Peripheral targets are exemplified by the skin, muscle and tendons.
the intermediate zone and ventral part of the spinal cord. Small nociceptive neurons convey thermal and potentially harmful (painful; mechanical, thermal and chemical) stimuli from free nerve endings in the skin. The central projections of these neurons terminate in the dorsal horn of the spinal cord. Medium-sized mechanoreceptive neurons respond to pressure and light touch in skin and send central axons to the deeper layers of the dorsal spinal cord.

How different stimuli are registered by sensory neurons is poorly understood. A few groups of proteins have been identified to be involved in these processes, but data from in vivo and in vitro experiments are variable and sometimes contradictory. The perhaps most studied group is the transient receptor potential (Trp) cation channel family that among the sensory neurons is expressed in nociceptors but also in other cell types (Caterina 2007). Members of this family have been implicated in the activation of nociceptors due to application of irritant chemicals (i.e. the mustard oil sensitive receptor TrpA1) and changes in temperature (i.e. the cool receptor TrpM8 and the heat sensitive capsaicin receptor TrpV1). Another family that is widely expressed in nociceptors and mechanoreceptors are the acid-sensing ion channels (ASICs). They can be activated by noxious pH (Waldmann 2001), but also seems to play a role in mechanosensation, although the molecular mechanism for this is not known (Lingueglia 2007).

1.4.3.2 A neural crest origin
DRG neurons are derived from the trunk neural crest (Marmigere and Ernfors 2007). The neural crest is thought to be induced from the dorsal neural tube by BMP and Wnt signalling. Cells exposed to these signals undergo an epithelial-to-mesenchymal transition, becoming highly motile. Between E8.5 and E10 trunk neural crest cells emigrate from the neural tube along a ventral path between the neural tube and the neighbouring somite. The migration of neural crest cells destined to become sensory neurons of the DRG can be divided into three waves. Early migrating cells show limited proliferation once inside the ganglion and give rise to a smaller fraction of larger neurons eventually expressing TrkB or TrkC. Later migrating neural crest cells proliferate and contribute to both the small neurons expressing TrkA and large neurons. Around E10 a third wave emanates to form the dorsal root entry zone boundary cap, a transient structure of cells at the entry point of sensory axons into the CNS (Topilko et al. 1994; Maro et al. 2004). These proliferative, multipotent (Hjerling-Leffler et al.
cells will generate a last wave almost exclusively giving rise to small TrkA positive neurons.

Neurogenins (Ngn) initiate neurogenesis in neural crest cells in two waves and bias them to the sensory lineage (Ma et al. 1999). Ngn2 starts to be expressed in a subset of migratory neural crest cells around E9.5, and this expression is not down-regulated until the DRG has coalesced. Ngn1, on the other hand, is turned on around E10 in postmigratory cells within the DRG promoting neurogenesis in late born cells.

1.4.3.3 Multiple roles for neurotrophins
The three main categories of DRG neurons, nociceptors, mechanoreceptors and proprioceptors, express during embryonic development TrkA, TrkB and TrkC, respectively. TrkB and TrkC, but not TrkA, mRNA has been detected already in neural crest cells (Martin-Zanca et al. 1990; Tessarollo et al. 1993). However, a study using antibodies failed to detect any of the Trk receptors in migrating neural crest cells at E9 (Farinas et al. 1998). Instead the Trk-receptors were first observed at E10-11, and only in cells showing a neuronal phenotype. Shortly after this, many innervation targets start to express the mRNA of the corresponding neurotrophins (Schecterson and Bothwell 1992). Ngf is detected in skin from E11.5 with a peak at E15.5. Bdnf is expressed broadly in skin at E11.5, but becomes restricted to sensitive areas such as the lips, tongue and digits at E15.5. Nt3 is also present in skin at E11.5 with a strong expression around developing vibrissae at E15.5. At the same time Nt3 is also expressed in myoblasts and later at E14.5, Nt3 can be detected in hair follicles. Apart from innervation targets, Nt3 has also been observed along migratory routes for DRG axons (Patapoutian et al. 1999). Adding to the complexity, neurotrophin mRNA has also been shown to be expressed within the DRG, implying autocrine and/or paracrine functions (Ernfors et al. 1992; Schecterson and Bothwell 1992; Farinas et al. 1996).

In general, studies using knock-out mice have proven a good correlation between the proportion of DRG neurons that express a certain Trk receptor and their dependence on activation of this receptor by its cognate ligand for survival. In Ngf−/− and TrkA−/− mice about 70% of the neurons are lost, showing a reduction in the number of small neurons corresponding to the nociceptive and thermoceptive population. In line with this, heterozygote animals exhibit decreased responses to painful stimuli (Crowley et al. 1994; Smeyne et al. 1994). In TrkB−/− and Bdnf−/− mice about 30% of the DRG neurons
are lost (Klein et al. 1993; Ernfors et al. 1994a; Jones et al. 1994; Liebl et al. 1997). In Nt4\(^{-/-}\) mice a small neuronal death (14\%) has been reported (Liu et al. 1995), and D-hair innervation of hair follicles is lost postnatally (Stucky et al. 1998).

For TrkC\(^{-/-}\) and Nt3\(^{-/-}\) mice, the situation is more complex. While TrkC\(^{-/-}\) mice show a modest reduction in neuronal numbers (~35\%), DRG of Nt3\(^{-/-}\) mice have been analyzed showing 55-79\% loss of neurons (Ernfors et al. 1994b; Farinas et al. 1994; Liebl et al. 1997; Tessarollo et al. 1997). This indicates a more general dependence on NT3 than expected from the TrkC expression. Lost neurons in Nt3\(^{-/-}\) mice have indeed been shown to belong to all three subtypes of sensory neurons, with the severest effect on parvalbumin expressing proprioceptors (Ernfors et al. 1994b; Airaksinen and Meyer 1996; Farinas et al. 1998). How and when NT3 acts on the TrkA and TrkB populations is controversial. Excessive neuronal death in Nt3\(^{-/-}\) mice has been demonstrated both early (~E11-12) and later (~E13.5-15.5) in DRG development affecting survival of precursors and/or neurons (ElShamy and Ernfors 1996; Liebl et al. 1997; Farinas et al. 1998). It has been proposed that these effects are mediated either through the cognate receptor TrkC in a common earlier population or through TrkA and TrkB. Afferents lost in TrkC\(^{-/-}\) and Nt3\(^{-/-}\) mice during embryonic development normally innervate the limbs and the mice display movement deficits and extreme postures and also lack tendon organs and muscle spindles. Postnatally a loss of Merkel cells and their innervation by slowly adapting mechanoreceptors with A\(\beta\) fibres conduction velocity also takes place (Airaksinen et al. 1996).

Since neurotrophins are so essential for neuronal survival in the sensory system, it has not been possible to use the conventional knock-out animals mentioned above to study later importance of neurotrophin actions in DRG. A couple of different genetic approaches have been taken to circumvent this. The neurotrophin survival dependence of peripheral neurons is regulated by the proapoptotic gene Bax, and the generation of double null mutants for Bax and NGF or TrkA has allowed the study of NGF/TrkA signalling beyond neuronal survival (Patel et al. 2000). Additional functions have thus been added showing that NGF is required for the actual target innervation and expression of markers such as ion channels, GPCRs and Ret in nociceptive neurons (Patel et al. 2000; Luo et al. 2007). Another strategy that has proven the importance of neurotrophin signalling for phenotypic maturation and central connectivity is the genetic replacement of TrkA with TrkC (Moqrich et al. 2004). The surviving
presumptive TrkA-expressing neurons adopt a proprioceptive phenotype, indicating that neurotrophin signalling can specify sensory neuron subtypes. In addition, the central projections of these neurons terminate in the ventral instead of the dorsal horn of the spinal cord.

1.4.3.4 GDNF family ligands in late nociceptive development
In two populations of DRG neurons, Ret replaces the expression of a Trk receptor. In a subpopulation of mechanoreceptive neurons, Ret is already present at E11.5 and TrkB subsequently gets down-regulated and is almost abolished in these cells at E14.5 (Kramer et al. 2006). Later, in a subpopulation of nociceptors, Ret expression is induced around E17 and reaches adult levels at P7.5, while TrkA is being down-regulated before P21 (Molliver et al. 1997). This population is further characterized by the lack of expression of neuropeptides and the ability to bind isolectin B4 (IB4), in contrast to the peptidergic population that continues to express TrkA (Silverman and Kruger 1988; Molliver and Snider 1997; Molliver et al. 1997; Bennett et al. 1998). The induction of Ret expression in nociceptors, as well as expression of Gfra1 and Gfra2 is under the control of TrkA (Luo et al. 2007). In turn Ret becomes important for the extinction of TrkA. GFRα1-3 are expressed in DRG during development and adulthood, but the reports on distribution of these co-receptors vary. In neonatal mice Gfra3 is the most widely distributed mRNA, followed by Gfra2 and Gfra1 (Baudet et al. 2000). In P14 mice it has been shown that virtually all Ret expressing neurons also co-express at least one GFRα, and that Gfra2 is most widely present in the Ret population, followed by Gfra1 and Gfra3 (Luo et al. 2007). In adult mouse, approximately 80% of the IB4-positive neurons express GFRα2 (Lindfors et al. 2006b). The majority of GFRα2-positive neurons has unmyelinated axons (~70%) and expresses the purinergic receptor P2X3 (85%). It has further been shown that GFRα3 protein is expressed in 20% of adult DRG neurons and that it is co-expressed with Ret (82%), the capsaicin receptor TrpV1 (99%) and, surprisingly, TrkA (80%) (Orozco et al. 2001). In correspondence to the expression of these GFL receptors in nociceptive neurons, innervation targets, such as the epidermis and whisker follicles, express Gdinf, Nrtn and Artn (Fundin et al. 1999; Golden et al. 1999; Elitt et al. 2006).

Analysing the phenotypes in DRG of GFL, GFRα receptor and Ret knock-out mice has demonstrated that signalling through Ret is not primarily involved in survival of the neurons, but more the trophic state, target innervation and phenotypic maturation of a
subtype of nociceptors. A neuronal loss (23%) has only been clearly reported for P0 
Gdnf⁻/⁻ mice (Moore et al. 1996). In addition to this loss, the remaining neurons showed 
a reduced average size. Gfra1⁻/⁻ mice however, do not display any neuronal loss and no 
hypotrophy has been reported (Cacalano et al. 1998; Enomoto et al. 1998). In Nrtnt⁻/⁻ 
mice the number of Gfra2 expressing neurons is reduced by 45% in the adult 
(Heuckeroth et al. 1999). It is however, not clear if this is due to neuronal death or just 
lost expression. In Gfra2⁻/⁻ mice, neurons do not die, but are reduced in size with about 
30% (Lindfors et al. 2006b). They also show a 70% reduction in epidermis innervation, 
but no changes were apparent in central innervation as assessed by the presence of IB4 
binding in inner lamina II of the spinal cord. Despite the expression of GFRα3 in DRG, 
both Gfra3⁻/⁻ and Artn⁻/⁻ mice have apparently normal DRG with maintained neuronal 
numbers (Nishino et al. 1999; Honma et al. 2002). Since Ret knock-out mice die just 
after birth, they are not amendable to studying postnatal development in DRG. 
Recently, a Ret conditional knock-out mouse, with Ret-deficiency in neural crest 
derivatives, was published (Luo et al. 2007). In these mice, the DRG are completely 
devoid of Ret expression and neuronal numbers are intact, but the soma size is reduced 
for non-peptidergic neurons. Similar to the Gfra2⁻/⁻ mice, epidermis innervation is 
severely compromised, while central innervation appears normal. This study also 
showed that Ret signalling is necessary for the full maintenance of Gfrα1 and Gfrα2 
expression. This further strengthens the possibility that the loss of Gfra2 in the NRTN 
knock-out is likely to be just a loss of expression and not neuronal death. In addition, Ret 
was also shown to be needed for the full expression of TrpA1 and members of the Mas- 
related GPCR (Mrgpr) family, which are markers of mature nociceptors. Some of the 
knock-out mice mentioned above have not been thoroughly examined in terms of 
marker expression, innervation and physiological significance and much remains to be 
done to fully appreciate GFL signalling in DRG. See chapter 2.3 for a discussion on the 
physiological importance of Ret in development.
2 RESULTS AND DISCUSSION

2.1 PAPER I

NT3 signalling through all three Trk receptors is a generally accepted physiological process, based on data in vitro and assumptions from in vivo experiments. However, whether NT3 actually binds and activates its non-cognate receptors in vivo is not clear. To directly address this, we took a genetic approach, using a set of genetically manipulated mice, analysing in vivo whether NT3 is able to signal through TrkB in the cochleovestibular system. The cochleovestibular system is unique in the sense that it expresses both TrkB and TrkC in most neurons during development.

2.1.1 NT3 exclusively signals through TrkC in vivo

Neuronal counts in E18 mice (Table 1 and 2, paper I), confirmed previously published data showing greater neuronal loss in the vestibular ganglion, than in the cochlear ganglion of Bdnf^{-/-} mice, and a complete rescue in the cochlear ganglion as compared to a partial rescue in the vestibular ganglion in Bdnf^{-Nt3/Nt3} mice (Ernfors et al. 1994a; Agerman et al. 2003). The incomplete rescue in the vestibular ganglion could reflect that a proportion of the vestibular ganglion neurons do only express TrkB, or that NT3 signalling through TrkB does not give the same effect on survival. However, removing TrkB in the Bdnf^{-Nt3/Nt3} background had no effect on neuronal numbers, indicating that the rescue caused by introduction of NT3 was not mediated by TrkB, thus excluding NT3 signalling through TrkB. Predictably in contrast, all neurons were lost in double mutant mice lacking TrkC in the Bdnf^{-Nt3/Nt3} background, showing the importance of TrkC for NT3 signalling. So, despite the opportunity to signal through TrkB (levels of expression unchanged, Fig. 1, paper I) NT3 exerts its effect through TrkC. Another system, where NT3 is not able to signal through TrkB, is the geniculate ganglion that innervates the papillae. These neurons express TrkB and when Bdnf is removed 50% of the neurons die, but additional Nt3 expression in Bdnf^{-Nt3/Nt3} mice can not rescue the lost neurons (Agerman et al. 2003). The explanation for that NT3 in these two systems does not signal through TrkB, could be the fact that in vivo TrkB is not a receptor for NT3, or that certain factors are present that prevent NT3 from signalling through TrkB, such as expression of p75NTR or splicing of TrkB, making it less susceptible to NT3.
However, it is not only the spatiotemporal expression patterns that determine the neurotrophin/Trk interactions.

### 2.1.2 NT3/TrkC signalling is qualitatively different from BDNF/TrkB signalling

Even if *Nt3* replacing *Bdnf* can support neuronal survival in the cochleovestibular system, the rescue of innervation is not as efficient (Fig. 3, paper I). In these knock-in animals innervation is more abundant than in *Bdnf* +/- mice, but most of the fibres are stalled in the sub-epithelial layer, not reaching the hair cells. Indeed these mice do not regain functionality and display deficits in balance coordination, like the *Bdnf* +/- mice (Ernfors et al. 1994a; Agerman et al. 2003). This demonstrates a qualitative difference in signalling between NT3/TrkC and BDNF/TrkB, and a non-redundancy for final target innervation. This could also explain the incomplete rescue of neurons in the vestibular ganglion of *Bdnf*[^Nt3/Nt3] mice, if reaching the hair cells is a requirement for sufficient trophic support. Overall, afferent innervation seems to be more severely affected than efferent innervation as judged by a double staining for p75[^NTR] (afferents) and βIII-tubulin (afferents and efferents) (Fig. S1, paper I). It is however notable that the fibres only positive for βIII-tubulin also are reduced, implicating that efferent innervation is affected as well. This could be due to the fact that many efferents synapse on afferents.

### 2.2 PAPER II AND III

Adaptor proteins recruit different signalling complexes to activated RTKs, inducing signalling pathways that overlap to a certain extent. The contribution of an adaptor bound to an RTK for the biological response of the trophic factor have often been investigated *in vitro* by overexpression of the adaptor, silencing by small interfering RNAs or fusion to the receptor. In paper II and III we have taken advantage of mutated Ret receptors that preferentially recruit Dok (Fig. 1 and 2, paper III) Shc or Frs2 (Lundgren et al. 2006) to Y1062. These rewired receptors minimize effects from competing adaptors at Y1062 to an otherwise intact cellular milieu.
2.2.1 Two separate phosphorylated tyrosines on Ret cooperates in mediating a migrational response

Ret has been shown to be involved in migration, both in vitro and in vivo. By using pharmacological inhibitors in gut explant cultures, pathways such as PI3K, MAPK and Jnk have been implicated in GDNF-induced migration (Natarajan et al. 2002; Asai et al. 2006). We show that binding of Frs2 to Y1062 in Ret is necessary for directional migration, while binding of Shc is not (Fig. 1, paper II). In addition to this, we show that Y981, which recruits and activates Src, is required for a full migratory response after ligand stimulation (Fig. 3A and B, paper II). The migrational response seen below Ret is thus dependent on the recruitment of molecules to two different phosphorylated tyrosines. Furthermore, phosphorylation of MAPK and the participation of focal adhesion kinase (FAK) is needed downstream of Frs2 and Src for migration (Fig. 2C, 3E). MAPK are known to be activated in migration and Src and FAK are involved in the turn over of focal adhesions, which is necessary for migration.

2.2.2 Frs2 recruitment of Ret concentrates the receptor into discrete membrane foci thereby altering downstream signalling

While the recruitment of both Shc and Frs2 to activated Ret leads to the phosphorylation of FAK (Fig. 3C, paper II), only Frs2-mediated signalling will localize clusters of FAK to cell protrusions and focal adhesions (Fig. 4A-P, paper II). Likewise does activation of Src by Ret, require Frs2 binding at Y1062 (Fig. 2A, H, I paper II). This is in line with that Ret previously has been shown to interact with Frs2 and Src mainly inside lipid rafts, while Shc interaction takes place outside (Tansey et al. 2000; Encinas et al. 2001; Paratcha et al. 2001). Together with our data, this suggests that Frs2 recruits Ret to lipid rafts where it can interact with Src and cause a local phosphorylation of FAK that is important for migration. In support, Frs2-recruiting Ret receptors are present in foci of cell protrusions in difference from Shc-recruiting receptors that are more evenly distributed in the cell (Fig. 5, paper II).

2.2.3 Subcellular localization of Ret is dependent on adaptor binding

We show that a Ret receptor that preferentially recruits Dok induces prolonged MAPK signalling, but can not activate Akt (Fig. 3A, paper III). MAPK activation via Dok has been described before (Uchida et al. 2006), however that study also found a prolonged phosphorylation of Akt. The difference in Akt activation could be attributed to the use
of different cell lines (SK-N-MC vs TGW) or the fact that the overexpression of Dok in Uchida et al. does not exclude the interaction of other adaptors with Y1062. In contrast, our Dok binding receptor does not interact with Shc (Fig. 2C, D, G, paper III), an adaptor normally associated with signalling through Akt (Besset et al. 2000; Hayashi et al. 2000; Lundgren et al. 2006). Overexpressed Dok could also theoretically participate further down the signalling pathway(s), implying that the Akt activation is not necessarily a consequence of Dok binding to Y1062.

Ret signalling through Dok has been implicated in neurite out-growth, but apart from activating MAPK and Rap1, not much is known about the mechanism (Grimm et al. 2001; Crowder et al. 2004; Uchida et al. 2006). We show that short stimulation of Dok-recruiting receptors induce massive formation of microspikes and the redistribution of Ret to focal points at the tip of these protrusions (Fig. 3B-D). The redistribution of Ret can also be seen for wild-type receptors, but not for receptors with a silencing mutation of Y1062 (Y1062F). Thus it seems that the recruitment of Dok to Y1062 is important for the localization of the receptor and the formation of these priming structures.

2.3 PAPER IV

Ret is expressed in the DRG, both during development and in the adult animal. It was recently demonstrated in Ret-deficient pups that Ret expression during development is necessary for induction and/or maintenance of different markers of the sensory neurons, some of which are implicated in sensory function (Luo et al. 2007). However as these animals die around P14, the adult phenotype was never investigated, including the physiological role of Ret. We have generated another strain of Ret conditional knock-out animals that lack Ret in approximately 50% of the IB4-binding nociceptive neurons (Fig. 1, paper IV) and are viable and fertile.

2.3.1 Ret is important for expression of markers in the adult DRG

In a screen of sensory marker expression in adult DRG of Ret-deficient animals, we found changes in mRNA levels for different classes of markers, including Trp cation channels, ASICs and members of the Mrgpr family, implying that Ret could be important for the functionality of sensory neurons in the adult animal (Fig. 2, paper IV). Most of the markers have lowered levels of expression such as TrpA1, Asic2a and
$\text{MrgprB4}$, indicating that Ret is involved in induction or maintenance of their expression. The changes in $\text{TrpA1}$ and $\text{MrgprB4}$ expression levels are similar to the results obtained by Luo et al., considering that the reduction of Ret is 50%. Overexpression of $\text{Gdnf}$ in the skin has been shown to increase the amount of ASIC2a expressed in DRG, while overexpression of $\text{Artn}$ gives the opposite effect (Albers et al. 2006; Elitt et al. 2006). In conjunction with our data this implies a role for Ret signalling in the regulation of this transcript.

The unchanged levels of peptidergic markers such as calcitonin gene-related peptide, substance P and TrkA indicates that the peptidergic lineage of nociceptors is unaffected by the removal of Ret (Fig. 2, paper IV). In the previous study by Luo et al., they showed that the down-regulation of TrkA at P14 was incomplete, suggesting that Ret is important for the extinction of TrkA in the non-peptidergic population. Since TrkA levels are unaffected in our adult animals this implies a delay in the extinction, when Ret is not expressed.

2.3.2 Ret is involved in the developmentally dynamic regulation of $\text{MrgprA3}$ expression

In contrast to the other markers, $\text{MrgprA3}$ is expressed in the adult Ret conditional knock-out, while the transcript is missing in the control animals, implying that Ret in this case is involved in the down-regulation. This result is unexpected as MrgprA3 has been shown to be expressed in the adult (Dong et al. 2001). We used quantitative real-time PCR, while Dong et al. have used in situ hybridization and this might account for the difference. $\text{MrgprA}$ transcripts are highly homologous, which might affect the specificity of the probes. Also, using in situ hybridization the transcript was shown to be down-regulated at P14 in Ret-deficient animals implying that Ret is important for induction and/or maintenance (Luo et al. 2007). GDNF stimulation of P2 DRG explants is able to up-regulate the transcript for MrgprA3 (Fig. 4B, paper IV). This capacity is lost at P12 and correlates well with the developmental expression of MrgprA3 (Fig. 4A, paper IV). It thus appears that Ret is also involved in the induction of the transcript.

2.3.3 Ret deficiency leads to mechanical and thermal sensitization

Ret-deficient animals show increased sensitivity to mechanical stimulation of the hind paw by von Frey filaments and heat stimulation of the tail, but not to heat or cold stimulation of the hind paw (Fig. 5, paper IV). The unchanged response to heat
stimulation of the paw, but reduced response latency of the tail, is similar to the phenotype observed in Gfra2\(^{−/−}\) mice (Lindfors et al. 2006b), implicating Nrtn/GFRα2 signalling through Ret in the development of normal noxious thermosensation. The sensitization of Ret conditional knock-out animals is moderate, which could reflect the fact that expression of Ret is only lost in about half of the non-peptidergic nociceptors. Full penetrance of the phenotype might demand a complete reduction.

Peptidergic nociceptors have traditionally been considered important contributors to inflammatory pain and non-peptidergic nociceptors the strongest candidate for mediating neuropathic pain (Snider and McMahon 1998). After carrageenan-induced inflammation both Ret conditional knock-out and control animals develop mechanical allodynia and heat hyperalgesia in the hind paw (Fig. 6A, paper IV). The hypersensitivity is, however, more profound in the conditional knock-out. After photochemically-induced sciatic nerve injury, no difference in response to mechanical stimulation was detected (Fig. 6B, paper IV). If non-peptidergic nociceptors are responsible for neuropathic pain, this implicates that Ret does not influence this phenomenon.

The enhanced sensitivity in Ret-deficient animals could be due both to physiological changes caused by altered expression patterns of functional markers and morphological changes of innervation. Explaining the increased heat and mechanical sensitivity based on changes in TrpA1, Asic2a, MrgprB4 and MrgprA3 expression is not straightforward. Reduction of ASIC2a could contribute to the change in mechanical sensitivity, since ASICs have been implicated in this process. Furthermore lowered ASIC2a expression could alter the pH sensitivity of the neuron. pH is indeed lowered in inflammation and this could be important for the sensitization seen during inflammation. Although the function for Mas-related GPCRs is not known, it is interesting that MrgprA3 is up-regulated in the more sensitive Ret conditional knock-out animals. Generating MrgprA3 knock-out animals could prove interesting in determining the physiological role of this postnatally expressed GPCR. Further characterization of the expression of physiologically important markers in Ret-deficient animals might give a more complete picture. Based on other results (Lindfors et al. 2006b; Luo et al. 2007), it could be assumed that our animals have reduced target innervation in the skin. It would be of interest to know if the nerve fibres that are not in contact with the target do still detect stimuli or if they are silent. If they do not transmit
(proper) signals to the CNS, this could cause an imbalance in the perception with input only from peptidergic nociceptors.

2.4 CONCLUSIONS

How functionality and specificity is achieved in a cell after binding of a neurotrophic factor is only a partly understood process. In this thesis we show that

- Even if a certain neurotrophin and Trk receptor have spatiotemporally overlapping expression patterns the ligand might not activate the receptor.
- Different neurotrophin/Trk pairs can evoke qualitatively different responses within the same sensory neuron and are thus not always functionally redundant.
- Adaptor binding is not only important for coupling a receptor to certain intracellular signalling pathways, but also for the cellular localization of the receptor and this together generates the specific biological response.
- More than one phospho-tyrosine on an activated RTK can cooperate to generate a full biological response.
- GDNF family ligand signalling through Ret is important for the complete differentiation and maturation of nociceptive neurons and for the generation of a proper physiological response to painful stimuli.

In conclusion this thesis contributes to the elucidation of how specificity in neurotrophic factor signalling is achieved and the definition of new roles for neurotrophic factors in development.
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