GDNF FAMILY RECEPTORS IN PERIPHERAL TARGET INNERVATION AND HORMONE PRODUCTION

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Academic dissertation

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SELECTED ABBREVIATIONS

AChE	acetylcholine esterase
ARTN	artemin
BMP	bone morphogenetic protein
cAMP	cyclic adenosine monophosphate
CaR	calcium-sensing receptor
C-cells	clear cells
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CT	calcitonin
DRG	dorsal root ganglion
E	embryonic day
ERK	extracellular signal-regulated kinase
GDNF	glial cell line-derived neurotrophic factor
GFL	GDNF family ligand
GFRα	GDNF family receptor alpha
GPI	glycosyl phosphatidyl inositol
HSCR	Hirschsprung's disease
IB_4	isolectin B_4
KO	knockout
MAPK	mitogen-activated protein kinase
MEN2	multiple endocrine neoplasia type 2
mRNA	messenger RNA
MTC	medullary thyroid carcinoma
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
NOS	nitric oxide synthase
NRTN	neurturin
NT-3	neurotrophin 3
P	postnatal day
$P2X_3$	ATP gated cation selective channel $2X_3$
PCR	polymerase chain reaction
PGP9.5	protein gene product 9.5
PI3-K	phosphatidylinositol 3-kinase
PNS	peripheral nervous system
PSPN	persephin
RET	Ret tyrosine kinase (*re*arranged during *t*ransfection)
RT-PCR	reverse transcription PCR
SCG	superior cervical ganglion
SP	substance P
TGF-β	transforming growth factor beta
TH	tyrosine hydroxylase
TRP	transient receptor potential
Tyr	Tyrosine
VAChT	vesicular acetylcholine transporter
VIP	vasoactive intestinal peptide
WT	wild-type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by Roman numerals (I-IV), as well as on some unpublished results.

I Rossi J, Herzig K-H, Võikar V, **Hiltunen PH**, Segerstråle M, Airaksinen MS (2003) Alimentary tract innervation deficits and dysfunction in mice lacking GDNF family receptor α2. J Clin Invest 112(5): 707-716.

II **Hiltunen PH**, Airaksinen MS (2004) Sympathetic cholinergic target innervation requires GDNF family receptor GFRα2. Mol Cell Neurosci 26: 450-457.

III **Lindfors PH**, Võikar V, Rossi J, Airaksinen MS (2006) Deficient nonpeptidergic epidermis innervation and reduced inflammatory pain in glial cell line-derived neurotrophic factor family receptor α2 knock-out mice. J Neurosci 26(7): 1953-1960.

IV **Lindfors PH**, Lindahl M, Rossi J, Saarma M, Airaksinen MS (2006) Ablation of persephin receptor glial cell line-derived neurotrophic factor family receptor α4 impairs thyroid calcitonin production in young mice. Endocrinology 147(5): 2237-2244.

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF) family ligands: GDNF, neurturin (NRTN), persephin (PSPN) and artemin (ARTN), signal through a receptor complex consisting of a glycosyl phosphatidyl inositol (GPI)-linked subunit (GFRα1-4) and the transmembrane receptor tyrosine kinase Ret. The GDNF family factors can support the survival of various peripheral and central neuronal populations *in vitro* but are required *in vivo*, particularly for the development of the peripheral nervous system (PNS). GDNF has important functions also outside the nervous system, especially in kidney development. Activating mutations in the RET gene cause tumours in neuroendocrine cells, whereas inactivating mutations in RET are found in patients with Hirschsprung's disease (HSCR) characterized by loss of ganglionic cells along the intestine.

The aim of this study was to examine the *in vivo* functions of GFRα2 and GFRα4 using knockout (KO) mice. Mice lacking GFRα2 grow poorly after weaning and have deficits in parasympathetic and enteric innervation. This study shows that impaired secretion of the salivary glands and exocrine pancreas contribute to growth retardation in GFRα2-KO mice. These mice have a reduced number of intrapancreatic neurons and decreased cholinergic innervation of the exocrine pancreas as well as reduced excitatory fibres in the myenteric plexus of the small intestine. This study also demonstrates that GFRα2-mediated Ret signalling is required for target innervation and maintenance of soma size of sympathetic cholinergic neurons and sensory nociceptive IB_4-binding neurons. Furthermore, lack of GFRα2 in mice results in deficient perception of temperatures above and below thermoneutrality and in attenuated inflammatory pain response. GFRα4 is co-expressed with Ret predominantly in calcitonin-producing thyroid C-cells in the mouse. In this study GFRα4-deficient mice were generated. The mice show no gross developmental deficits and have a normal number of C-cells. However, young but not adult mice lacking GFRα4 have a lower production of calcitonin in thyroid tissue and consequently, an increased bone formation rate. Thus, GFRα4/Ret signalling may regulate calcitonin production. In conclusion, this study reveals that GFRα2/Ret signalling is crucial for the development and function of specific components of the peripheral nervous system and that GFRα4-mediated Ret signalling is required for controlling transmitter synthesis in thyroid C-cells.

REVIEW OF THE LITERATURE

Introduction

The mammalian nervous system contains an enormous number of neural cells. During embryogenesis the neurons are organized into a functioning nervous system through a chain of developmental steps. This complex process involves committing to a neural fate, proper migration of the neurons into their destined positions, guiding growing neurites into target tissues and controlling the balance between survival and cell death. These developmental steps are orchestrated by various environmental cues, intrinsic factors and soluble factors secreted from target tissues. Much of this development is regulated by so-called neurotrophic factors. During embryogenesis the neurotrophic factors are involved in guiding the migration of neural precursors and axons towards their proper targets as well as in supporting the survival and differentiation of mature neurons. In adult animals, neurotrophic factors take part in regulating synaptic plasticity and tissue renewal. Furthermore, neurotrophic factors have functions outside the nervous system.

In most parts of the developing central and peripheral nervous system, neurons are produced in excess, with up to twice as many neurons produced as needed by the mature animal. However, during the period of programmed cell death the number is reduced to correspond to the actual requirement of the innervated tissue (Burek and Oppenheim, 1996). Neurons that are able to extend their projections to the correct target tissue receive trophic factors allowing them to survive. If a neuron fails to reach its target or does not receive a sufficient amount of neurotrophic factors, it will die. Neurotrophic factors are able to block the cell death program and can also activate specific survival pathways. Although several growth factors have neuronal survival effects, the traditional neurotrophic factors include three families: the neurotrophins (NGF, BDNF, NT-3 and NT-4/5) (Huang and Reichardt, 2001; Sofroniew et al., 2001), the neurokines (e.g. LIF, CT-1 and CNTF) (Sariola et al., 1994) and the GDNF family of neurotrophic factors (Baloh et al. 2000; Airaksinen and Saarma, 2002; Sariola and Saarma, 2003; Enomoto, 2005).

Glial cell line-derived neurotrophic factor family

GDNF family ligands

The GDNF family of neurotrophic factors comprises four members: GDNF (glial cell line-derived neurotrophic factor), neurturin (NRTN), artemin (ARTN) and persephin (PSPN). These molecules form a subgroup in the transforming growth factor beta (TGF-β) superfamily and contain seven conserved cysteine residues with the same relative spacing found in all members of the TGF-β family (Eigenbrot and Gerber, 1997). The mature GDNF family proteins are biologically active as glycosylated disulphide-bonded homodimers. They are synthesized as precursors (preproproteins) with an amino-terminal signal sequence that is cleaved on secretion and a prosequence that is cleaved from the mature polypeptide by a furin-like

endoproteinase at RXXR cleavage sites (Fig. 1) (Lin et al., 1993; Kotzbauer et al., 1996; Baloh et al., 1998b; Milbrandt et al., 1998).

Orthologs of the four GFLs are present in most vertebrate classes. But whereas bony fish genomes contain more than four GFL orthologs, one of the ligands, NRTN, is absent in clawed frog *Xenopus tropicalis* and another, PSPN, in the chicken genome. So far no GFL orthologs have been found in insects or other invertebrates. Thus, the time of origin of GFLs remains unclear, but the first GDNF family ligand presumably diverged from existing TGF-β-like proteins (reviewed in Airaksinen et al., 2006).

The founding member of this family, GDNF, was originally purified from a rat glial cell line based on its potent survival effect on embryonic midbrain dopaminergic neurons (Lin et al., 1993). Later, GDNF was shown to also promote the survival of several other central neuron populations, including spinal motoneurons (Henderson et al., 1994), locus coeruleus noradrenergic neurons (Arenas et al., 1995), basal forebrain cholinergic neurons (Williams et al., 1996) and cerebellar Purkinje cells (Mount et al., 1995). Furthermore, GDNF can support peripheral sensory, sympathetic, parasympathetic and enteric neurons (Buj-Bello et al., 1995; Heuckeroth et al., 1998; Forgie et al., 1999). Many areas of the central and peripheral nervous systems in developing and mature mice express GDNF mRNA (Trupp et al., 1995; Golden et al., 1999). It is also detected in several peripheral organs, such as the embryonic muscle wall of the gastrointestinal tract, kidney mesenchyme, developing skin and muscle, whisker follicles and dental mesenchyme (Trupp et al., 1995; Suvanto et al., 1996; Luukko et al., 1997; Sainio et al., 1997; Golden et al., 1999). Consequently, GDNF also functions outside the nervous system. For instance, it acts as a morphogen in kidney development and regulates the differentiation of adult spermatogonial stem cells (Meng et al., 2000; Sariola, 2001). GDNF protein expression has been detected at least in the gastrointestinal tract (Peters et al., 1998), kidney (Camassei et al., 2003), spinal cord and various brain areas (Kawamoto et al., 2000).

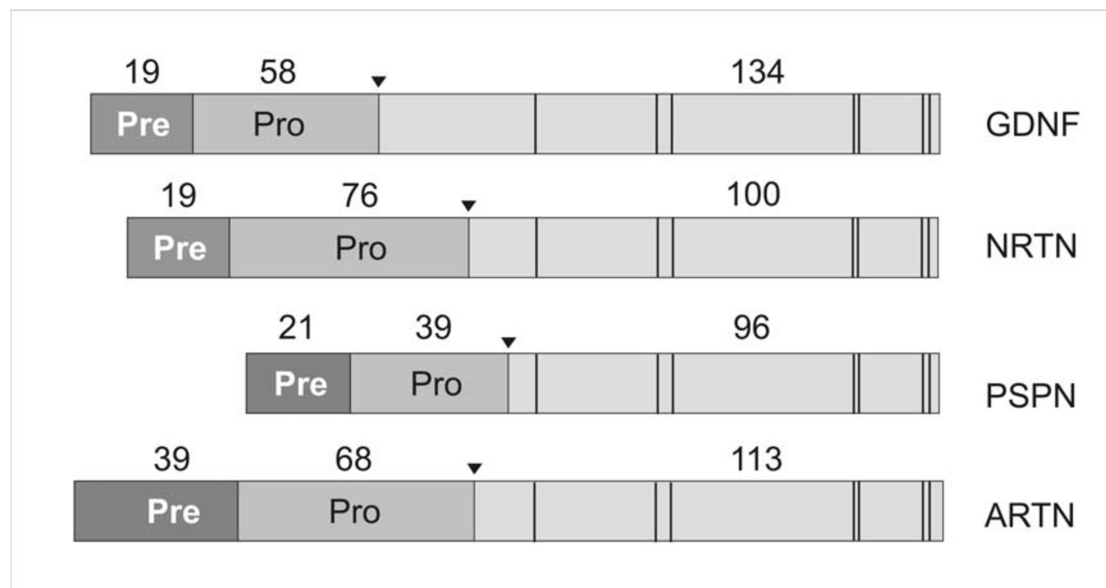

Figure 1. Schematic structure of GDNF family ligands. Shown are relative lengths (number of amino acids) of pre- and prodomains and the mature protein. Black lines mark positions of seven conserved cysteine residues and the RXXR cleavage site is indicated with arrowheads. Adapted from Airaksinen et al. (1999).

Because of its powerful neuroprotective effects, GDNF has raised interest as a potential therapeutic agent for treatment of several neurological diseases (reviewed in Airaksinen and Saarma, 2002). Encouraging results obtained from animal experiments set great expectations for GDNF in the treatment of Parkinson's disease. Indeed, GDNF has been shown to prevent cell death of dopamine neurons and even to promote functional recovery in many animal models of Parkinson's disease (Björklund et al., 2000; Kordower et al., 2000; Åkerud et al., 2001). Unfortunately, results from clinical trials have so far been contradictory. Although in an open-label trial, local delivery of GDNF directly into the putamen significantly improved motor skills of Parkinson's disease patients (Gill et al., 2003), a recent double-blind, placebo-controlled trial failed to confirm these results (Lang et al., 2006).

NRTN was found in a Chinese hamster ovary cell line as a survival factor for cultured sympathetic neurons (Kotzbauer et al., 1996). Mature mouse NRTN protein is about 45% identical to mouse GDNF and in addition to sympathetic neurons, it promotes the survival of some sensory neuron populations (Kotzbauer et al., 1996), enteric neurons (Heuckeroth et al., 1998), parasympathetic submandibular neurons (Cacalano et al., 1998), dopaminergic neurons (Kotzbauer et al., 1996), spinal motoneurons (Garces et al., 2001) and embryonic basal forebrain cholinergic neurons (Golden et al., 2003). Similarly to GDNF, exogenous NRTN is able to support dopamine neuron survival in mouse models of Parkinson's disease (Horger et al., 1998; Åkerud et al., 1999), but unlike GDNF it does not induce axonal growth or hypertrophy. NRTN transcripts are detected within and outside the nervous system during embryonic development and in maturity, e.g. in the brain, skin, many exocrine glands, testis, kidney epithelium, circular muscle layer of the gastrointestinal tract, lungs and dental epithelium (Widenfalk et al., 1997; Luukko et al., 1998; Golden et al., 1999). In rat, NRTN protein has been localized by immunohistochemistry e.g. in salivary gland, small intestine and kidney (Xian et al., 1999).

Human mature PSPN is ~40% identical to GDNF and NRTN and was discovered using degenerate PCR. Like all other members of the GDNF family, it supports the survival of many central neurons from embryonic rats, including midbrain dopaminergic neurons, motoneurons (Milbrandt et al., 1998) and basal forebrain cholinergic neurons (Golden et al., 2003) *in vitro*. But unlike other GDNF family ligands, it has not supported any of the peripheral neurons examined (Milbrandt et al., 1998). *In vivo* PSPN may protect the brain from ischaemic insults since in mouse and rat models ischaemia-induced neuronal cell death could be prevented by exogenous PSPN (Tomac et al., 2002). Furthermore, in a mouse model of Parkinson's disease, the loss of dopamine neurons was prevented by grafting a neural stem cell line overexpressing PSPN to the striatal area (Åkerud et al., 2002). Two *Pspn* transcripts, an unspliced form and a functional form, are expressed at very low levels in various adult and embryonic tissues in rats (Milbrandt et al., 1998). PSPN-like immunoreactivity has been detected in human and rat brain (Quartu et al., 2005).

The fourth member of the GDNF family, ARTN was found using database searches based on homology to NRTN. It is the most distantly related member of the GDNF family, with 36% homology to GDNF at the amino acid level. ARTN can support the survival of sensory and sympathetic neurons in culture (Baloh et al., 1998b), and it is expressed along the migratory routes of sympathetic neuroblasts in smooth muscle cells of blood vessels and arteries (Nishino et al., 1999; Honma et al., 2002). ARTN was shown to prevent neuropathic pain and reverse the associated

morphological and neurochemical changes in an animal model (Gardell et al., 2003). ARTN can also support midbrain dopaminergic neurons in culture (Baloh et al., 1998b). However, only low levels of ARTN mRNA have been detected in embryonic and adult human brain e.g. in the basal ganglia (Baloh et al., 1998b).

GDNF family receptors

All GDNF family ligands can signal through a receptor tyrosine kinase Ret by binding first to a co-receptor (GFRα1-4) that is attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor (Fig. 2). Although GFRα receptors are usually bound to the plasma membrane, they can be cleaved by an unknown protease or phospholipase to produce soluble forms (Paratcha et al., 2001). GFRα receptors have an amino-terminal signal sequence for secretion and a carboxy-terminal hydrophobic domain for GPI linkage. GFRα1-3 are proposed to have a domain structure of three homologous cysteine-rich domains (D1-D3) joined together by less conserved adapter sequences (Airaksinen et al., 1999). In GFRα1, domains 2 and 3 have been shown to interact with both GDNF and Ret (Leppänen et al., 2004). Mammalian GFRα4 is however, smaller, lacking the first amino-terminal cysteine-rich domain (D1) (Lindahl et al., 2000). Each GDNF family ligand has a preferred GFRα co-receptor: GFRα1 for GDNF (Jing et al., 1996;Treanor et al., 1996), GFRα2 for NRTN (Baloh et al., 1997; Buj-Bello et al., 1997; Klein et al., 1997; Sanicola et al., 1997), GFRα3 for ARTN (Baloh et al., 1998a; Naveilhan et al., 1998; Widenfalk et al., 1998) and GFRα4 for PSPN (Enokido et al., 1998; Lindahl et al., 2000). At least *in vitro*, NRTN and ARTN bind weakly to GFRα1 and GDNF to GFRα2, but the physiological significance of this "cross-talk" is unclear (Airaksinen et al., 1999) (Fig. 2).

Orthologs of the four GFRα receptors are found in all vertebrate classes. GDNF receptor alpha like (GRAL), a distant homolog of GFRα, is also present in verebrates, but the binding partners for GRAL are so far unknown. Furthermore, a GPI-linked protein, Growth-arrest specific 1 (GAS1) may be distantly related to GFRα. GFR-like genes exist also in insects (rewieved in Airaksinen et al., 2006).

Gfra1 mRNA is expressed in several brain areas, the spinal cord and some peripheral ganglia and usually in a complementary pattern to GDNF in peripheral organs, such as the developing kidney and urethra, whisker follicles and dental epithelium, and in a subset of spermatogonia (Luukko et al., 1997; Nosrat et al., 1997; Sainio et al., 1997; Golden et al., 1999; Meng et al., 2000). GFRα1 protein has been detected in developing rodent gut where it becomes localized to enteric neurons (Worley et al., 2000; Gianino et al., 2003). GFRα1 immunoreactivity has also been found in human neuromuscular junctions and myelinated nerves (Hase et al., 1999), bovine Schwann cells (Hase et al., 2005) and in human trigeminal ganglion neurons (Quartu et al., 2006). Furthermore, many regions of rat central nervous system (CNS), including spinal motoneurons, substantia nigra and cerebellar cortex show GFRα1 immunoreactivity (Matsuo et al., 2000). *Gfra2* transcripts are detected throughout the developing and adult nervous system, including various brain areas, parasympathetic, sympathetic and sensory ganglia and the myenteric plexus of the gastrointestinal tract, but also in some peripheral organs like the developing tooth and testis (Baloh et al., 1997; Luukko et al., 1997; Widenfalk et al., 1997; Golden et al., 1999; Rossi et al., 1999). GFRα2 protein has been localized in parasympathetic nerve fibers in salivary and lacrimal glands (Rossi et al., 2000) and in both parasympathetic nerve fibers and

glia in endocrine pancreas (Rossi et al., 2005) and pelvic ganglion (Wanigasekara et al., 2004). GFRα2 protein expression is also detectable in the newborn and adult gut in mouse (Gianino et al., 2003). Furthermore, GFRα2 immunoreactivity has been detected in the hippocampal formation (Nanobashvili et al., 2000). Expression of *Gfra3* is mostly restricted to the peripheral nervous system during development, including sensory and sympathetic neurons and Schwann cells (Baloh et al., 1998a; Naveilhan et al., 1998; Widenfalk et al., 1998). GFRα3 protein is detected in a subpopulation of sensory neurons (Orozco et al., 2001). The mouse *Gfra4* gene is alternatively spliced during development in a tissue-specific manner, producing GPI-linked and transmembrane isoforms as well as transcripts with premature stop codons (Lindahl et al., 2000). Although *Gfra4* transcripts are found in many embryonic and adult tissues, including the nervous system and the testis, the splice form leading to a functional GPI-linked isoform of GFRα4 is expressed only in the juvenile thyroid and parathyroid glands in mouse. In the newborn mouse and adult mouse thyroid, parathyroid, pituitary intermediate lobe and adrenal medulla, a transmembrane isoform of GFRα4 is produced instead (Lindahl et al., 2000). Due to lack of a good GFRα4 antibody, the localization of GFRα4 protein has not been determined before this study (IV).

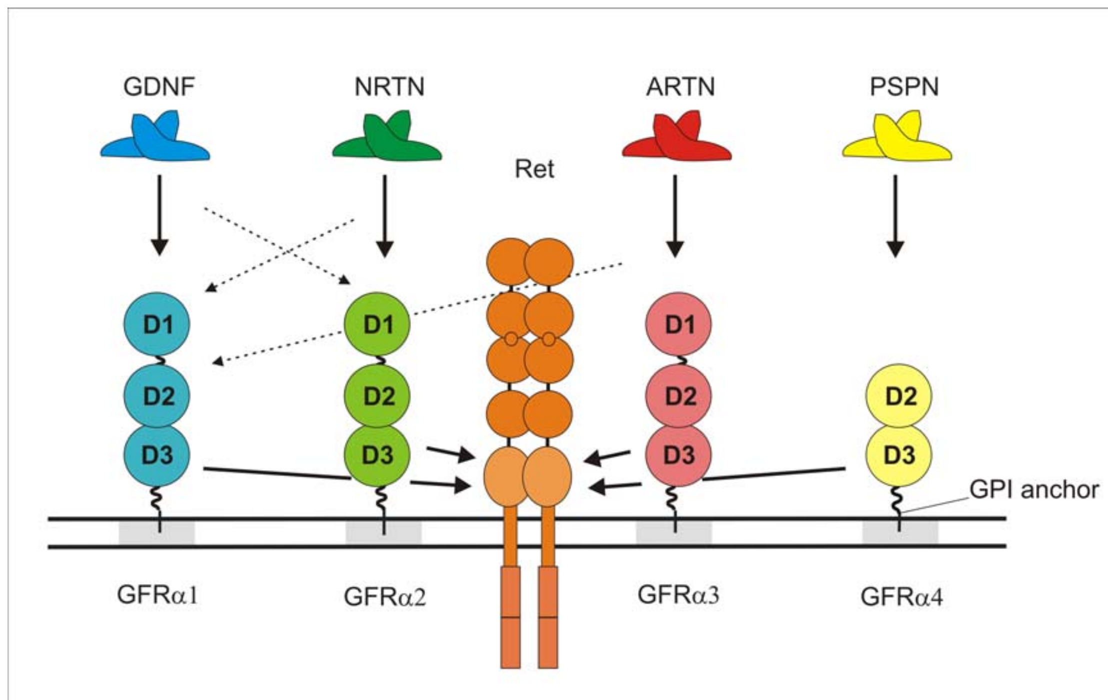

Figure 2. GDNF family ligand (GFL) receptor complex and interactions. All GFLs activate the transmembrane tyrosine kinase receptor Ret via their preferred GFRα receptors (solid arrows). Weak "cross-talk" has been observed *in vitro* (dotted arrows). The mammalian GFRα4 lacks the first cysteine-rich domain D1. The GFRα receptors are attached to the cell membrane with a GPI anchor. Adapted from Airaksinen and Saarma (2002).

Ret (*re*arranged during *t*ransfection), the common signalling receptor for GDNF family ligands, 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). Ret oncogene encodes a single span transmembrane tyrosine kinase with an extracellular ligand binding domain containing four cadherin-like repeats and a Ca^{2+} binding site (Anders et al., 2001), a cysteine-rich domain (Takahashi et al., 1988; Takahashi et al., 1989; Iwamoto et al., 1993; Kuma et al., 1993) and an intracellular domain with tyrosine kinase activity. Alternative splicing of *Ret* gives rise to two major Ret isoforms that differ in their C-terminal tail. The long isoform of 1114 amino acids (RET51) contains two additional tyrosine residues in positions 1090 and 1096 within the carboxyl terminus (Tahira et al., 1990) (see also Fig. 4). The short isoform of 1072 amino acids (RET9) is important for enteric innervation and renal development (de Graaff et al., 2001), whereas RET51 is required for the growth and metabolism of mature sympathetic neurons (Tsui-Pierchala et al., 2002a). *Ret* orthologs are present in all vertebrate classes and Ret-like genes have been found also in insects. The *Drosophlia* D-ret and vertebrate Ret genes are expressed in many analogous tissues suggesting similar functions. The ligand of D-ret, however, is not known (reviewed in Airaksinen et al., 2006).

Ret mRNA is usually expressed similarly to one or several GFRα co-receptors and in a complementary pattern to their ligands; in the intestine, for example, *Gfra1* and *Ret* are expressed in the ganglionic plexuses of the developing gastrointestinal tract, while *Gdnf* is expressed in the muscle layers (Nosrat et al., 1997). Ret gene and protein expression is detected in tissues derived from the neural crest, such as sympathetic, enteric and sensory neurons, thyroid C-cells and adrenal chromaffin cells (Tsuzuki et al., 1995; Belluardo et al., 1999), and in tumours of neural crest origin (Takaya et al., 1996). In the central nervous system (CNS), Ret mRNA and protein expression is prominent in dopamine neurons and spinal motoneurons (Tsuzuki et al., 1995; Nosrat et al., 1997; Trupp et al., 1997; Golden et al., 1999). In addition, Ret transcripts and protein are observed in the developing rodent kidney and the gastrointestinal tract (Nosrat et al., 1997;Golden et al., 1999;Tsuzuki et al., 1995;Worley et al. 2000).

RET-dependent GDNF family signalling

The original model of GDNF signalling proposed that a GDNF dimer first binds to either a monomeric or dimeric GFRα1, and the resulting GDNF/GFRα1 complex interacts with two Ret molecules to induce their homodimerization and tyrosine phosphorylation (Fig. 3) (Jing et al., 1996). However, at least some Ret molecules seem to be pre-associated with GFRα1 before GDNF binding occurs (Eketjäll et al., 1999). This model requires both Ret and GFRα in the same cells (*in cis*) and indeed often their mRNA expression patterns overlap. However, *Gfra* receptors are much more widely expressed in the nervous system than *Ret* (Trupp et al., 1997) suggesting, that GFRα receptors can signal independently of Ret. Furthermore, GFRα receptors that can be cleaved from the cell surface to work as soluble forms might present the ligand to Ret located in the membrane of another cell (*in trans*) (Fig. 3) (Trupp et al., 1997).

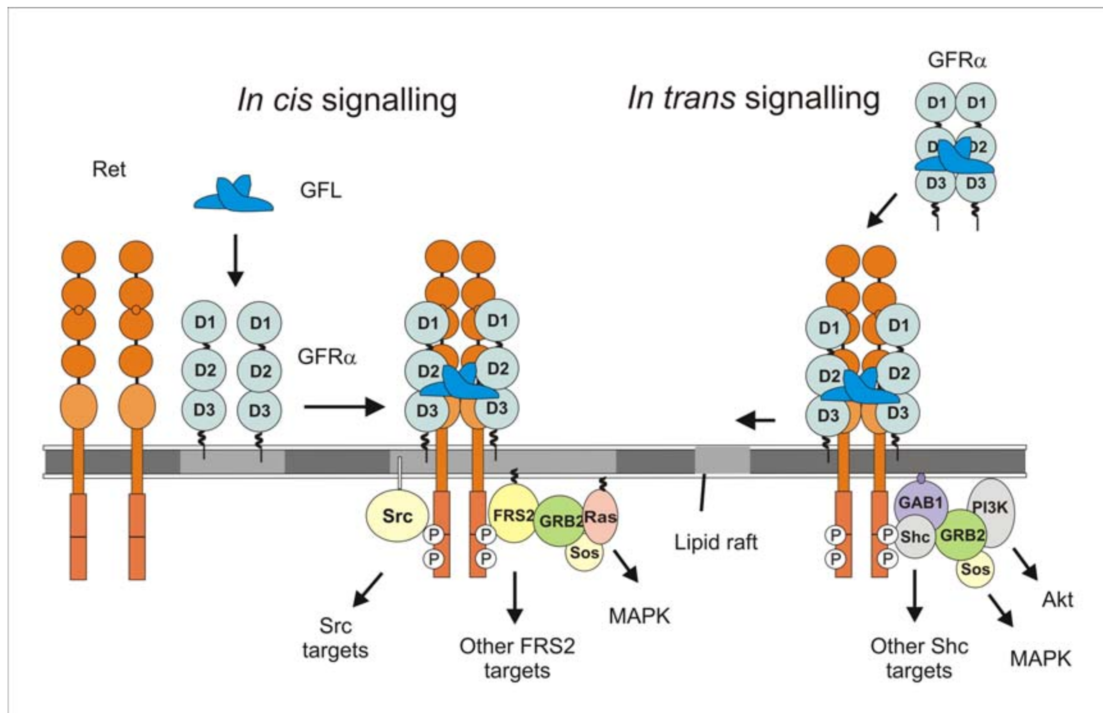

Figure 3. GDNF family signalling in lipid rafts (*in cis*) and outside rafts (*in trans*). GFRα receptors are located in the lipid rafts. Inactive Ret is localized outside rafts, but GFL binding to the GFRα receptor recruits Ret to the raft. Recruitment promotes the binding of lipid-anchored adaptor proteins to the receptor complex and the activation of Src. Activated Ret associates preferentially with FRS2 inside the rafts, but outside the rafts Ret is associated with Shc. Adapted from Airaksinen and Saarma, 2002.

Lipid rafts are specialized signal transduction platforms that can sort signalling molecules, allowing them to interact with each other within the raft and preventing interactions with proteins that are excluded from the raft (Simons and Ikonen, 1997). The rafts consist of dynamic assemblies of cholesterol and sphingolipids on the outer leaflet of the plasma membrane surrounded by a fluid disordered phase of the lipid bilayer. Several protein groups, such as GPI-linked proteins, cytoplasmic Src family kinases, cholesterol-linked and palmitylated proteins and some transmembrane proteins have a high affinity to rafts (Simons and Toomre, 2000). The GPI anchor of GFRα receptors localizes them to lipid rafts of the plasma membrane (Poteryaev et al., 1999). Inactive Ret is situated outside the rafts, but GDNF binding to GFRα1 localizes Ret to the lipid rafts and triggers an association with intracellular signalling molecules such as Src (Tansey et al., 2000) (Fig. 3). Soluble GFRα1 is also able to recruit Ret to the lipid rafts, and the *in trans* signalling could occur for instance in peripheral nerves, where Schwann cells produce GFRα1 while sympathetic and sensory axons express Ret (Trupp et al., 1997; Paratcha et al., 2001). Moreover, soluble GFRα receptors may function to increase the ligand specificity of Ret signalling (Worley et al., 2000). Possibly, during different developmental stages neurons require different signalling mechanisms: a GFL-GFRα gradient during cell migration but a fixed local source during axonal growth and branching (Airaksinen and Saarma, 2002). However, contrary to GFRα1, GFRα4 is unable to recruit Ret to rafts upon ligand stimulation, even though PSPN/GFRα4 complex can induce phosphorylation of Ret (Yang et al., 2004). Possibly GFRα1 and GFRα4 interact differently with other cell surface proteins (Yang et al., 2004). Furthermore, the significance of lipid rafts has been questioned, since most of the evidence relies on

biochemical extraction studies; so far lipid rafts have not been visualized in living cells (Munro et al., 2003).

Binding of the GFL/GFRα complex brings two transmembrane Ret molecules into contact and allows transphosphorylation of specific cytoplasmic tyrosine residues to occur. The phosphorylated tyrosine residues (Tyr) of Ret serve as high-affinity binding sites for a number of intracellular signalling proteins (Fig. 4). For example, Tyr905, Tyr1015, Tyr1062 and Tyr1096 represent docking sites for growth factor receptor-bound proteins 7 and 10 (GRB7/10), Phospholipase Cγ (PLCγ), Src homology domain containing protein C (Shc) and GRB2, respectively. Tyr1062 is the binding site for many adaptor proteins including Shc, fibroblast growth factor receptor substrate 2 (FRS2), downstream of tyrosine kinase 4/5 (Dok4/5), insulin receptor substrate 1/2 (IRS1/2) and Enigma (Takahashi, 2001; Kurokawa et al., 2003; Ichihara et al., 2004). Like other tyrosine kinase receptors, Ret can activate a variety of signalling pathways, including p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), RAS/extracellular signal-regulated kinase (ERK), ERK5 and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (Fig. 4). All of these pathways are activated mainly through tyrosine residue 1062 (Hayashi et al., 2000). After Shc binds to Tyr1062, it further associates with GAB1/2 and GRB2 adaptor proteins, leading to activation of PI3K/Akt signalling pathway, and with GRB2/SOS complex to activate RAS/ERK signalling pathway. RAS/ERK and PI3K pathways result in the activation of transcription factors cAMP responsive element (CREB) and nuclear factor κB (NFκB), respectively. Binding of FRS2 to Tyr1062 also leads to the RAS/ERK activation via GRB2/SOS adaptor protein complex (Kurokawa et al., 2003; Ichihara et al., 2004). How p38MAPK, JNK and ERK5 pathways are activated via Tyr1062 is still unclear (Ichihara et al., 2004).

The Ras/ERK pathway activation has been shown to be crucial for survival and differentiation of neurons and for neurite outgrowth (Califano et al., 2000; De Vita et al., 2000). Furthermore, it may be involved in branching of the ureteric bud (Fisher et al., 2001). The PI3K pathway is also necessary for neuron survival and proliferation as well as for lamellipodia formation, which is needed for neurite outgrowth (Takahashi, 2001; Fukuda et al., 2002). GDNF-induced lamellipodia formation can be regulated by another signalling pathway, too. Protein kinase A (PKA) has been shown to phosphorylate serine 696 of Ret following intracellular cyclic adenosine monophosphate (cAMP) level increase (Fig. 4). This phosphorylation is important for Rac activation and lamellipodia formation (Fukuda et al., 2002). Intracellular cAMP concentration is regulated through synthesis of cAMP by adenylyl cyclase and breakdown by cyclic AMP phosphodiesterase. Trimeric GTP-binding proteins (G proteins) activate adenylyl cyclase via G-protein-linked receptors. This suggests cross-talk between Ret and G-protein-coupled receptors in modulating cytoskeletal structures (Fukuda et al., 2002). The PLCγ signalling pathway is known to regulate the intracellular Ca^{2+} level via inosotol (1,4,5)-trisphosphate (IP_3) (Airaksinen et al., 1999). Ret signalling also triggers activation of Src-family kinases, which induce neurite outgrowth and neuronal survival (Tansey et al., 2000). The major binding site for Src appears to be Tyr981 in Ret (Fig. 4) (Encinas et al., 2004).

Although stimulation of Ret activates various survival-promoting pathways, in the absence of a ligand Ret can induce apoptosis in certain cell lines. This pro-apoptotic effect of Ret can be prevented by adding GDNF and may represent a form of ligand-independent Ret signalling (Bordeaux et al., 2000). The stimulation of Ret triggers different signalling pathways inside and outside rafts. Thus, altering the

location of Ret may provide additional diversity to intracellular GDNF family signalling. Activated Ret interacts with lipid-anchored adaptor protein FRS2 only inside rafts and with soluble Shc mainly outside rafts (Fig. 3). Intriguingly, both of these proteins require the same docking site in Ret (Tyr1062), suggesting that competition between these two adaptor proteins for the same site in Ret could affect the variation observed in intracellular signalling (Paratcha et al., 2001).

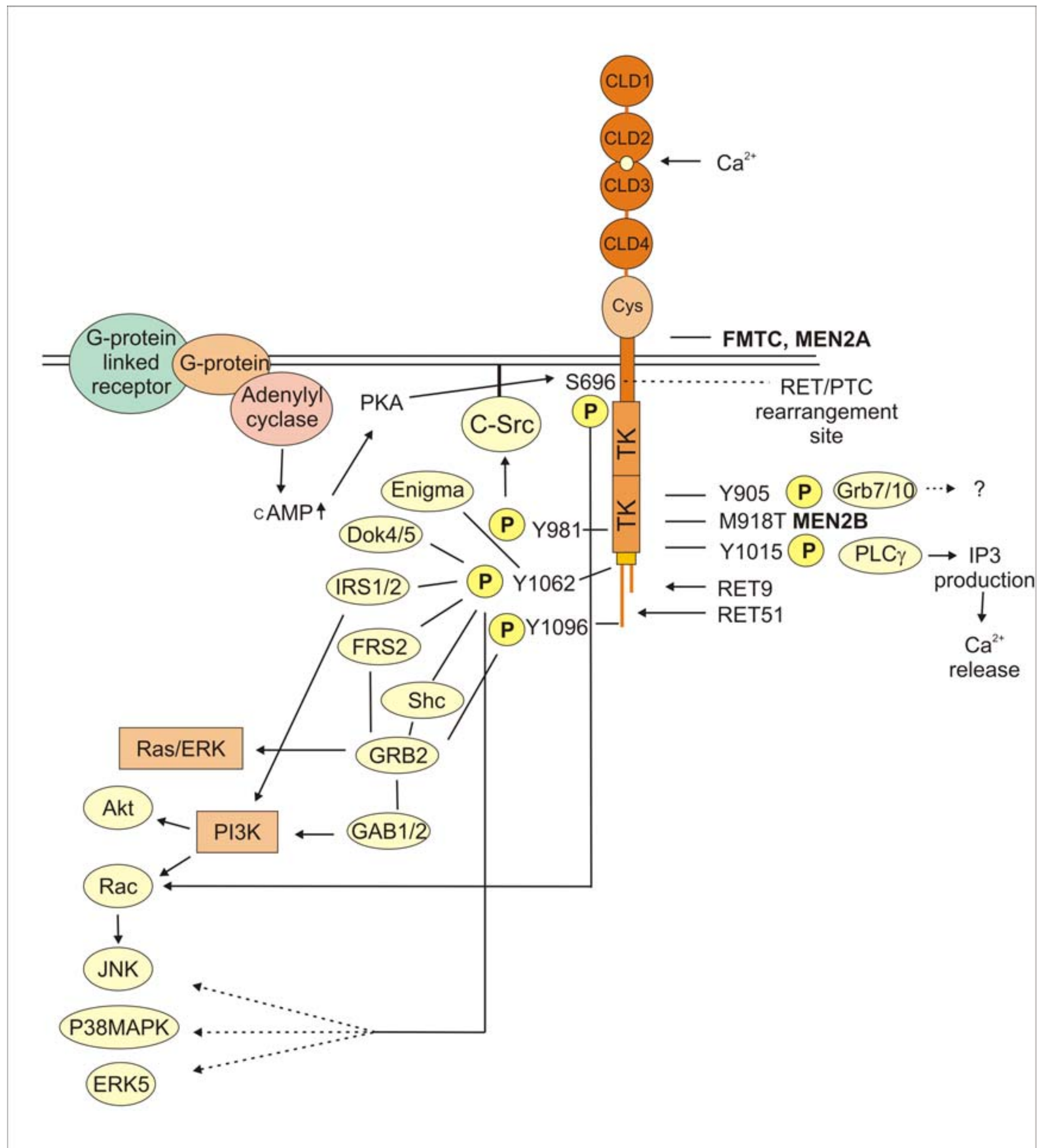

Figure 4. Intracellular pathways followed by Ret activation. Transphosphorylation of intracellular tyrosine residues (Y) in Ret provide binding sites for various adaptor proteins, which specify the down-stream signalling pathways. The most common mutation sites of RET in human cancer syndromes MEN2A, MEN2B and FMTC as well as the rearrangment site in PTC (see below) are indicated. Adapted from Lindahl (2004).

Current data indicate that GDNF signalling is more complex than the original model by Jing et al. (1996) suggests. The simplified pathway by which GFLs activate Ret via GFRα co-receptors cannot explain all of the biological activities of GFLs. Cross-talk with other growth factors and their specific receptors appears to occur. Ample evidence suggests that GDNF requires the cooperating factor TGF-β to exert its trophic activity in peripheral and some central neurons (Krieglstein et al., 1998; Schober et al., 1999; Peterziel et al., 2002); TGF-β may help the ligand to recognize its receptor by clustering the GFRα1 molecules to the lipid rafts (Peterziel et al., 2002). Furthermore GDNF signalling requires cell-surface associated heparan sulphate glycosaminoglycans. Without heparan sulphate, Ret phosphorylation does not occur and GDNF fails to induce axonal growth and scattering of epithelial cells (Barnett et al., 2002). Binding of GDNF to heparan sulphate may serve to concentrate the growth factor in the vicinity of its high affinity GFRα receptors and Ret (Barnett et al., 2002). Interestingly, mice lacking heparan sulfate 2-sulfotransferase (an enzyme essential in the synthesis of heparan sulphates) resemble Ret-, GFRα1- and GDNF-deficient mice as they all lack kidneys (Bullock et al., 1998). Cooperation between GDNF family and neurotrophin signalling has also been detected.

Postnatally, sympathetic neurons lose their nerve growth factor (NGF) dependency for survival, instead requiring NGF for soma and neurite growth and to assume a mature neurotransmitter phenotype. During this transition period Ret phosphorylation is greatly increased in these cells. This increase does not, however, require GFLs or GFRα receptors but is induced by NGF. Activation of tropomyosin receptor kinase A (TrkA, the high-affinity NGF receptor) by NGF induces phosphorylation of the Ret long isoform independently of either GFLs or GFRα receptors (Tsui-Pierchala et al., 2002b). Recently, GAS1, a DNA synthesis preventing tumor suppressor protein, was proposed to serve as a competitive receptor for GFLs (Schueler-Furman et al., 2006). GAS1, which is localized to lipid rafts, exhibits homology to GFRα receptors, and the expression patterns of GAS1 and GFRα receptors overlap. Schueler-Furman et al. (2006) suggested that competition between GAS1 and GFRα co-receptors may in part determine the fate of the cells towards apoptosis or cell survival.

RET-independent GDNF family signalling

Ret-independent signalling of GDNF family factors had been suspected since GFRα receptors are expressed widely in many tissues without Ret (Trupp et al., 1997). GDNF binding to GFRα1 has subsequently been found to activate Src-family kinases as well as the ERK/MAPK and PLC-γ pathways and the transcription factor CREB in cells that do not express Ret (Poteryaev et al., 1999; Trupp et al., 1999). By contrast, the NRTN/GFRα2 complex seems unable to signal in the absence of Ret (Pezeshki et al., 2001). The finding that exogenous GDNF can increase branching in kidney explants from Ret-deficient mice led to the discovery of an alternative signalling receptor, Met receptor tyrosine kinase (Popsueva et al., 2003). GDNF could activate Met in a cell line lacking endogenous Ret, but the activation was not mediated by direct binding of GFRα1/GDNF to Met but via Src family kinases. The *in vivo* role of GDNF/Met signalling remains unclear (Popsueva et al., 2003). Another candidate for an alternative GDNF family signalling receptor is neural cell adhesion molecule (NCAM), which is widely co-expressed in, for example, the brain and Schwann cells with GFRα receptors. When GFRα1 is present, GDNF can bind NCAM with high

affinity leading to activation of tyrosine kinase Fyn and focal adhesion kinase FAK in the cytoplasm. Furthermore, GFRα1 is able to interact with NCAM and downregulate NCAM mediated cell adhesion even in the absence of GDNF (Paratcha et al., 2003). *In vitro* GDNF is able to stimulate Schwann cell migration via NCAM, independently of Ret (Paratcha et al., 2003).

Because the evidence for Ret-independent signalling has been obtained almost entirely with cell culture systems its physiological relevance remains unclear. To study this issue, mice lacking all Ret-independent GFRα1 expression were generated by expressing *Gfra1* under the control of *Ret* promoter in GFRα1-null background (Enomoto et al. 2004). These so-called *cis*-only mice had no deficits in regions where *trans*-signalling has been proposed *in vitro*, including kidney, enteric and motorneurons as well as regenerating nerves. Furthermore, no evidence was found to support the existence of GDNF/GFRα1/NCAM signalling *in vivo* (Enomoto et al., 2004). However, GFL/GFRα/NCAM signalling might have a role in regulating synaptic plasticity in the brain since NCAM -/- mice and GDNF+/- mice have similar cognitive defects (Cremer et al., 2000; Gerlai et al., 2001). Furthermore, GFRα1 mediated GDNF signalling was found to promote *in vivo* the differentiation and migration of cortical GABAergic neurons independently of both Ret and NCAM, suggesting alternative transmembrane signalling components (Pozas and Ibanez, 2005).

Derivatives of the neural crest: peripheral nervous system and neuroendocrine cells

The vertebrate neural crest is a transient stem cell population that originates from the dorsal neuroectoderm at the point of fusion of the neural tube, the organ rudiment that consecutively forms the CNS. Neural crest progenitors continue to divide as they migrate, following stereotyped routes, until they reach and colonize their final destinations. The neural crest progenitors are able to differentiate into cell types as diverse as pigment cells, connective tissue, facial cartilage and bone, and such neuroendocrine cells as adrenal chromaffin cells and thyroid C-cells and into neurons and glia of the PNS (see Fig. 5). While migrating, the initially pluripotent neural crest cells become exposed to a sequence of environmental cues which successively limit their developmental potential. A number of signals have been identified that bias the differentiation of neural crest cells along distinct lineages, including members of the Wingless (Wnt), fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) families. These instructive signals in turn activate the expression of a network of transcription factors that restrict the cellular fate by activating and suppressing particular genetic programmes (Sanes et al., 2006). For instance, bone morphogenetic proteins (BMPs) trigger a neurogenesis programme in autonomic neurons and induce the expression of transcription factors Mash1 (a mouse homologue of the *Drosophila* achaete-scute proneural gene) and Phox2b (paired-like homeobox 2a), which then regulate the expression of Phox2a (Lo et al., 1998;Pattyn et al., 1999). Furthermore, Phox2a together with Phox2b is able to induce the expression of Ret (Pattyn et al., 1999).

The PNS has somatic and autonomic divisions, both of which have motor and sensory components. Somatosensory neurons, which innervate the skin, muscle and joints, provide information about the outside environment and limb and muscle position, whereas viscerosensory neurons convey information about functioning of the

visceral organs. The motoneurons innervating voluntary skeletal muscles are sometimes also considered part of the PNS, although their cell bodies lay in the CNS and they are not derived from the neural crest. The autonomic nervous system controls involuntary visceral functions and can be further divided into three distinct divisions: sympathetic, parasympathetic and enteric. The sympathetic and parasympathetic divisions often display antagonistic effects and together are responsible for preserving the animal's internal homeostasis in a changing environment. The sympathetic nervous system participates in the response of the body to stress, whereas the parasympathetic acts to conserve the body's resources and restore homeostasis. The enteric nervous system controls the function of the smooth muscle of the gut and other digestive organs. The postganglionic parasympathetic neurons use acetylcholine as their neurotransmitter, whereas most postganglionic sympathetic neurons secrete noradrenaline. While many organs including the exocrine and endocrine glands, cardiac muscle, bladder and genitals receive input from both the sympathetic and the parasympathetic system, some targets receive innervation only from the sympathetic branch. These include the sweat glands, the adrenal medulla, the piloerector muscles of the skin and most blood vessels. Various brain regions influence the output of the autonomic nervous system usually via the hypothalamus, which integrates the received information (Dodd and Role, 1991).

Somatic sensory nervous system

Cell bodies of the somatic sensory neurons relaying information from the trunk and limbs are located in the dorsal root ganglia (DRG) lateral to the spinal cord, while sensory neurons relaying information from the facial area are situated in the cranial ganglia. The neurons in the sensory ganglia are bimodal: they send one axon to the peripheral tissues, and the other axon relays information from the periphery to the dorsal horn of the spinal cord or to the brainstem. The sensory neurons are specialized functionally, with different receptors for the detection of innocuous mechanical stimuli, such as light touch, vibration and pressure, and for the detection of painful stimuli and temperature. Low-threshold mechanoreceptors process external stimuli by activating specialized cutaneous and subcutaneous receptors at the body surface. So-called proprioceptors located in muscles, joints and other deep structures monitor mechanical forces that are generated internally. Neurons responsible for the perception of innocuous mechanical stimuli are of large diameter and are associated with rapidly conducting myelinated axons (Aα and Aβ fibres). Nociceptive neurons detect painful thermal, mechanical (high threshold) or chemical stimuli. Nociceptive axons terminate in unspecialized free nerve endings in the epidermal and dermal layers of the skin, and, being only lightly myelinated (Aδ fibres) or completely unmyelinated (C fibres), conduct relatively slowly. Pain can be separated into early perception of sharp pain, carried by Aδ fibres, and a later perception of a duller, burning quality, which is mediated by C fibres. Many nociceptors tend to respond to thermal, mechanical and chemical stimuli and are therefore said to be polymodal, while others have more specialized response properties (Meyer et al., 2006).

The neural crest cells that immigrate into the sensory DRG arise from the trunk neural crest (Fig. 5). Activation of Wnt signalling is required to direct the multipotent neural crest cells towards a sensory lineage (Lee et al., 2004). The migration of the sensory precursors occurs in two waves, producing to distinct subsets of sensory neurons. The first wave occurs in the DRG between E9.5 and E11.5, giving

rise to large-diameter sensory neurons, including mechanoreceptive neurons that express brain-derived neurotrophic factor (BDNF) receptor TrkB and proprioceptive neurons that express neurotrophin 3 (NT-3) receptor TrkC. The second wave occurs between E10.5 and E13.5 and forms the small-diameter nociceptive neurons that express NGF receptor TrkA. Transcription factors of the basic helix-loop-helix (bHLH) family, namely neurogenin 1 and 2, are required to generate the different subsets of sensory neurons (Ma et al., 1999). While the first wave of progenitors belongs to the early migrating neural crest cells, the second wave of progenitors originates at least in part from boundary cap cells. These cells arise from ventrally migrating neural crest cells and define the points at which axons pass into or out of the spinal cord (Maro et al., 2004). The sensory neurons that invade the cranial sensory ganglia, including trigeminal ganglia, arise from the neural crest and neurogenic ectodermal placodes (Baker and Bronner-Fraser, 2000).

Majority of sensory DRG neurons in rodents require NGF for survival during embryonic development (Silos-Santiago et al., 1995). Animals deprived of NGF/TrkA signalling have reduced sensitivity to painful stimuli at birth. About 70-80% of DRG neurons, including all nociceptors, are missing in these mice. Postnatally, however, half of the small neurons downregulate TrkA and upregulate Ret, the common GDNF family receptor (Molliver et al., 1997). The small-diameter DRG sensory neurons that mediate nociceptive and thermal responses can be divided into two populations based on anatomical, biochemical and physiological properties. About half are postnatally NGF-dependent (~40% in mice), express TrkA and synthesize such neuropeptides as calcitonin gene-related peptide (CGRP) and substance P. These peptidergic neurons project to lamina I and the outer region of lamina II in the spinal cord dorsal horn (Snider and McMahon, 1998). Other nociceptors (~30-50%, depending on species) lack neuropeptide expression, instead binding the plant isolectin B_4 (IB_4), expressing Ret and projecting to the inner region of lamina II of the spinal cord (Molliver et al., 1997). Most of these IB_4-binding, non-peptidergic neurons also express purinoceptor $2X_3$ ($P2X_3$), which is thought to be important in mediating the nociceptive actions of adenosine triphosphate (ATP) (Vulchanova et al., 1998). The difference between TrkA and Ret-expressing nociceptor populations suggests that they have distinct functional properties (Snider and McMahon, 1998). Recent studies imply that the peptidergic and non-peptidergic polymodal nociceptor populations also differ in their skin innervation patterns (Zylka et al., 2005) and ascending pathways to the brain (Braz et al., 2005). Pain consists of both a sensory and an emotional component. IB_4 nociceptors predominantly project to limbic regions of the brain, and thus, are more likely to contribute to the motivational, affective dimensions of the pain experience. The peptidergic population on the other hand may be more important for the discriminative aspects of pain, e.g. localizing the stimulus and verifying whether the stimulus is thermal or mechanical (Braz et al., 2005). Furthermore, the two populations may differ in their intracellular sensitization mechanisms since a protein kinase Cϵ signalling pathway, which is important in inflammation, was specifically activated in IB_4-binding nociceptors (Hucho et al., 2005).

Sensory receptors in the free nerve endings are activated by physical stimuli, such as temperature and pressure, and are able to convert the physical stimulus into chemical and electrical signals. Transient receptor potential (TRP) receptors are temperature-sensitive ion channels that are located within the free nerve endings in the skin and are activated by distinct physiological temperatures. Capsaicin, the hot ingredient of chili peppers, evokes a sensation of burning pain, and the molecular cloning of the capsaicin receptor has led to the identification of the first vanilloid

receptor TRPV1 (transient receptor potential vanilloid 1). TRPV1 is also activated by temperatures above 43ºC, a temperature most mammals perceive as noxious (Caterina et al., 2000; Davis et al., 2000). It is expressed in a subset of peptidergic and isolectin IB_4-binding neurons in the rat, but in the mouse only a few IB_4-positive neurons express TRPV1 (Zwick et al., 2002). Based on sequence identity, other thermosensitive members of the transient receptor potential (TRP) family of ion channels have been cloned and together they are able to detect a wide range of temperatures.

TRPV2 is predominantly expressed in the myelinated medium- to large-diameter sensory neuron population, and it responds to extremely hot temperatures with an activation threshold above 52ºC (Caterina et al., 1999). Innocuous warmth is transduced by receptors TRPV3 and TRPV4, which have activation temperatures of 34-38ºC and 27-34ºC, respectively (Patapoutian et al., 2003; Tominaga and Caterina, 2004). Both TRPV3 and TRPV4 are expressed in skin keratinocytes and are thought to communicate with the epidermal sensory endings to transduce thermal signals (Lee et al., 2005; Moqrich et al., 2005). Specific receptors for cool to noxious cold temperatures have also been discovered. Menthol receptor TRPM8 responds to moderately cool temperatures and is activated at ~25-28ºC, consistent with the cool feeling conveyed by menthol products. It is expressed in the sensory neuron fibres of the smallest diameter, but does not co-localize with any known nociceptive markers, such as CGRP, IB_4 and TRPV1 (Patapoutian et al., 2003). Another cold receptor TRPA1 (also known as ANKTM1) has a lower activation temperature than TRPM8. It is activated by cold at ~17ºC, a temperature that is reported to be painfully cold in humans. It is expressed in peptidergic cells within a subset of cells that co-express TRPV1. Nociceptors that can respond to both noxious hot and cold could provide a molecular explanation for the paradoxical cold phenomenon (Patapoutian et al., 2003; Story et al., 2003).

Many pain states in humans arise partly from actions of chemical mediators on nociceptors. A number of endogenous molecules, including bradykinin, serotonin, substance P, inflammatory cytokines, growth factors and small molecules such as nitric oxide (NO) and ATP are released from damaged cells but also from glial and immune cells. Many of these molecules can sensitize nociceptors, which means that the threshold for activation is reduced or the responsiveness of the nociceptors to suprathreshold stimuli is increased (McMahon et al., 2006). One candidate receptor for the transduction of nociceptive signals is $P2X_3$, an ATP-gated cation ion channel that is expressed particularly in the GDNF-responsive, IB_4-binding population of primary sensory neurons. Experiments done using $P2X_3$-deficient mice suggest that $P2X_3$ may mediate thermosensation as well as inflammatory pain (Cockayne et al., 2000; Souslova et al., 2000; Shimizu et al., 2005).

Virtually all of the IB_4-binding DRG neurons are *Ret* mRNA-positive and the majority also express *Gfra1* or *Gfra2* or both (Bennett et al., 1998, 2000). Only few IB_4-binding neurons express GFRα3. Most GFRα3-positive neurons belong to the peptidergic nociceptor population and are TrkA-positive (Orozco et al., 2001). Furthermore, *Ret* and *Gfra1* mRNA are expressed in a subpopulation of large-diameter DRG cells (Bennett et al., 1998, 2000). Somatosensory target areas, such as the epidermis, whisker follicles and tooth, express GFLs *Gdnf* and *Nrtn* (Luukko et al., 1997; Luukko et al., 1998; Fundin et al., 1999; Golden et al., 1999).

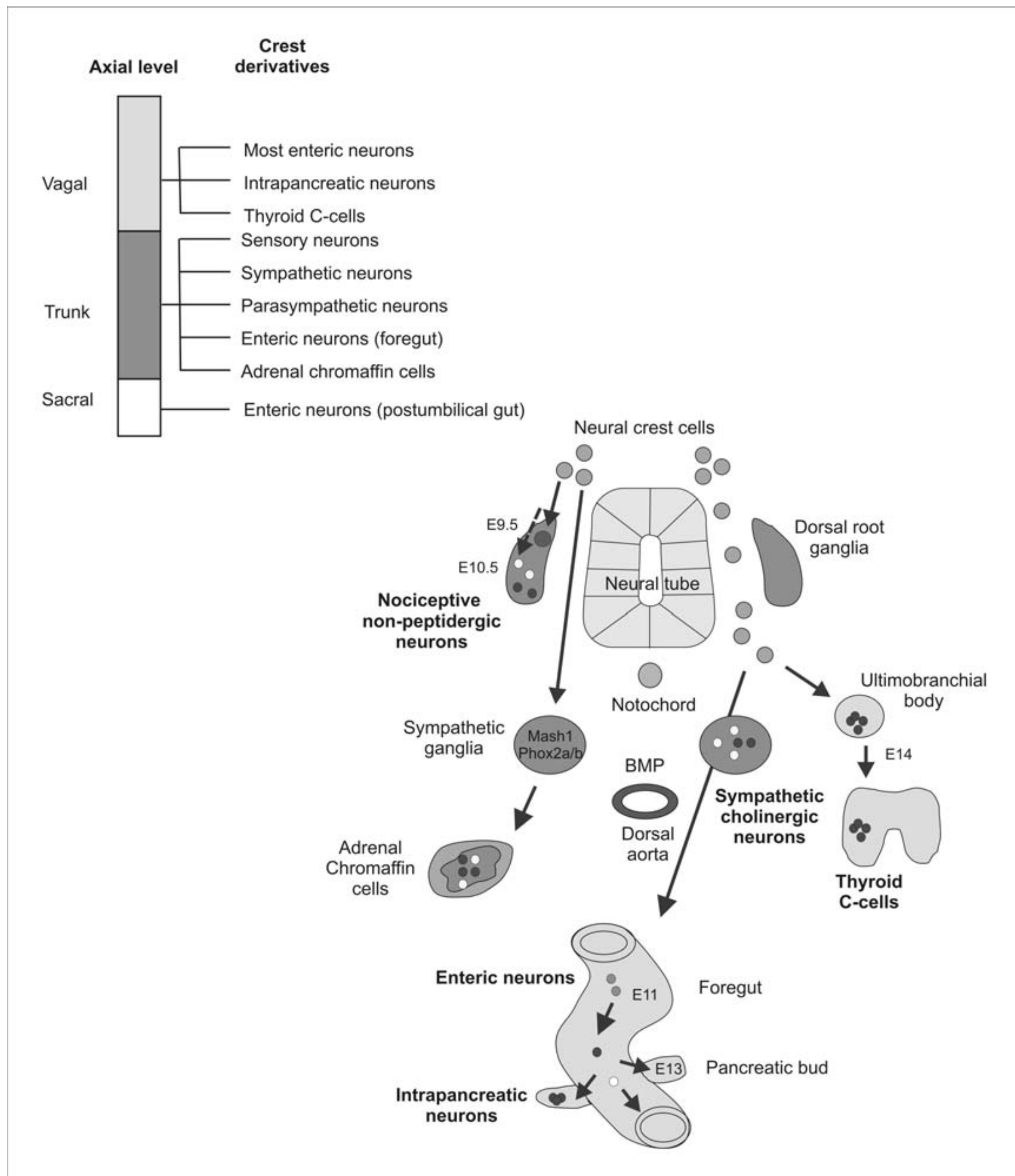

Figure 5. Derivatives of the trunk, vagal and sacral neural crest. Somatosensory and parasympathetic neurons as well as a population of enteric neurons are derived from the trunk neural crest. Sensory neurons colonize the dorsal root ganglia in two waves, generating large-diameter and then small-diameter neurons. Sympathetic neurons and adrenal chromaffin cells migrate together via the dorsal aorta, where secreted BMP induces the expression of Mash1 and Phox2a and Phox2b. Most enteric neurons and thyroid C-cells originate from the vagal neural crest. The thyroid C-cells migrate to the ultimobranchial body, which fuses with the thyroid rudiment to form the mature thyroid gland. Vagal-derived enteric precursors enter the foregut at E11 and colonize the gut along its length. At E13, the enteric precursors migrate to pancreatic buds, where they form intrapancreatic ganglia. Some enteric neurons also arise from the sacral neural crest.

Sympathetic nervous system and the adrenal gland

Preganglionic sympathetic neurons are situated at the thoracic and upper lumbar levels of the spinal cord and synapse onto postganglionic neurons in the sympathetic chain ganglia, which are situated on both sides of the spinal cord. The postganglionic neurons send their axons over relatively long distances to their peripheral targets. Sympathetic ganglia at the cervical and upper thoracic level, including the superior cervical ganglion (SCG) and the stellate ganglion, innervate various visceral glands as well as the heart, lungs, vascular smooth muscle, sweat glands and hair follicles. Some preganglionic fibres pass through the sympathetic chain without interruption to synapse on neurons of the prevertebral ganglia. These include the celiac ganglion and the superior and inferior mesenteric ganglia, which innervate the gastrointestinal system and provide sympathetic innervation to the bladder and external genitalia (Glebova and Ginty, 2005).

Postganglionic sympathetic neurons arise from the thoracolumbar level of the neural crest (Fig. 5). Together with cells immigrating to the adrenal medulla, these cells form the sympathoadrenal lineage. The sympathetic neuroblasts migrate ventrally near the dorsal aorta, where they coalesce to form the primordial sympathetic chain (reviewed in Anderson, 1993; Glebova and Ginty, 2005). BMPs secreted from the dorsal aorta induce (or maintain) the expression of transcriptional regulators, including Mash1, Phox2a and Phox2b in the sympathetic progenitors (Lo et al., 1998; Goridis and Rohrer 2002; Howard 2005). Mash1 and Phox2 induce the expression of the pan-neuronal and noradrenergic markers tyrosine hydroxylase (TH) and dopamine β-hydroxylase, which are components required for catecholamine biosynthesis from tyrosine (Guillemot et al., 1993; Lo et al., 1998; Pattyn et al., 1999). Some precursors in the primary sympathetic chain migrate further to generate the SCG and prevertebral ganglia and to give rise to adrenal chromaffin cells and some enteric neurons in the foregut (Fig. 5) (reviewed in Anderson, 1993; Glebova and Ginty, 2005).

The sympathetic neurons begin to protrude axons already during the formation of sympathetic ganglia and extend the axonal projections along blood vessels using them as intermediate routes on the way to their final target tissues. NT-3 is expressed in blood vessels and has been shown to induce proximal axon outgrowth but not axonal initiation (ElShamy et al., 1996; Francis et al., 1999). While the factors mediating axonal initiation are not known, a local autocrine growth factor loop involving hepatocyte growth factor (HGF) has been proposed (Glebova and Ginty, 2005). Although NT-3 is needed to mediate the proximal axon growth, the distal axonal extension and axonal branching require NGF, which is produced by sympathetic target tissues (Korsching, 1993). Furthermore, virtually all sympathetic neurons depend on target-derived NGF signalling via TrkA for survival (Francis et al., 1999; Glebova and Ginty, 2004). However, NT-3 does not appear to directly promote sympathetic neuroblast survival *in vivo*, even though NT-3-deficient mice exhibit 50% loss of SCG neurons (Ernfors et al., 1994). Instead, the reduced number of sympathetic neurons is likely due to impaired early axonal growth and consequent impaired ability to obtain target-derived NGF for survival (Kuruvilla et al., 2004).

While the majority of postganglionic sympathetic neurons are noradrenergic, a small subset uses acetylcholine as a neurotransmitter instead (reviewed in Ernsberger and Rohrer, 1999; Francis and Landis, 1999). In rodents, targets of these neurons include the eccrine sweat glands and periosteum, the connective tissue surrounding bone (Landis and Keefe, 1983; Schotzinger and Landis, 1988; Asmus et al., 2000).

Similarly to the majority of sympathetic neurons, the innervation of developing sweat glands is initially noradrenergic. After gland contact, these neurons downregulate noradrenergic properties, such as TH and catecholamine fluorescence, and begin to express cholinergic markers, including vasoactive intestinal peptide (VIP) and vesicular acetylcholine transporter (VAChT). This transmitter switch occurs during the first postnatal weeks in rodents (Ernsberger and Rohrer, 1999; Francis and Landis, 1999). Transplantation and co-culture experiments with sweat glands have shown that this conversion is mediated by a target-derived soluble factor (Schotzinger and Landis, 1988; Habecker and Landis, 1994; Guidry and Landis, 1998). Several neurokines, including leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and cardiotrophin 1 (CT-1), can promote cholinergic differentiation *in vitro* (Glebova and Ginty, 2005). However, in mice lacking LIF, CNTF or both, the cholinergic switch occurs normally (Francis et al., 1997). Still, signalling via the cytokine LIFRβ/gp130 receptor complex is required for the cholinergic switch in culture (Habecker et al., 1997; Francis and Landis, 1999; Duong et al., 2002). NT-3 and GDNF are other candidates for transmitter phenotype maturation, as they induce cholinergic properties in chick sympathetic neurons *in vitro* (Brodski et al., 2000, 2002)

The adrenal gland consists of the medulla and the mesodermally derived cortex, which produces glucocorticoids, aldosterone and androgens. The adrenal medulla is composed of sympathetic ganglion cells and two populations of chromaffin cells that produce catecholamines, either adrenalin or noradrenalin. The adrenal medulla receives preganglionic sympathetic innervation, and acetylcholine released from the sympathetic nerve endings stimulates the chromaffin cells to secrete catecholamines. Together with the sympathetic nervous system, the catecholamines constitute the "fight-or-flight" reaction by regulating numerous cardiovascular and metabolic processes (Randall et al. 1997). The neural crest progenitor cells arrive at the adrenal gland primordium and upregulate the expression of adrenalin-synthesizing enzymes phenyletholamine-N-methyl transferase (PNMT) and TH (reviewed in Anderson, 1993). The chromaffin cell maturation depends also on the function of transcription factors Mash1 and Phox2b, which are expressed by the sympathoadrenal progenitors (Huber et al., 2002, 2005) (see Fig. 5). Although an established idea of a common sympathoadrenal (SA) progenitor for adrenal chromaffin cells and sympathetic neurons exists, it has been challenged by recent studies. Novel evidence suggests that chromaffin progenitors are at least in part distinct from neuronal SA precursors before invading the adrenal gland (Unsicker et al., 2005)

Ret is expressed in the SA cells aggregating at the dorsal aorta (Pachnis et al., 1993). While mouse sympathetic neuroblasts express *Ret* abundantly at embryonic day 11.5 (E11.5) in the SCG and stellate ganglion, only a subpopulation of sympathetic neurons expresses *Ret* during late embryonic and neonatal development (Nishino et al., 1999; Enomoto et al., 2001). In the chicken, *Ret* is selectively expressed by the cholinergic subpopulation of the sympathetic chain ganglia (Ernsberger et al., 2000; Brodski et al., 2002), but it is not known whether *Ret* or any of the *Gfra* receptors are expressed by the cholinergic subpopulation of sympathetic neurons in mammals. *Gfra3* expression is abundant throughout the sympathetic nervous system during embryonic development but becomes undetectable in the adult (Nishino et al., 1999; Honma et al., 2002). *Gfra2* expression is regulated developmentally in mouse SCG, being first expressed diffusely by most cells at E12.5, then downregulated between E14.5 and E18.5 and again upregulated in a small

subpopulation of cells postnatally (Golden et al., 1999). Low levels of *Gfra1* are also expressed in SCG but in a pattern resembling non-neuronal cells (Baloh et al., 1997).

Of the GFLs, *Artn* is expressed in the vicinity of the developing sympathetic ganglia and in smooth muscle cells of blood vessels along the migratory routes of sympathetic neuroblasts but not in the final target tissues (Nishino et al., 1999; Enomoto et al., 2001; Honma et al., 2002). *Gdnf*, on the other hand, is expressed in many sympathetic target tissues (Trupp et al., 1995; Golden et al., 1999). Interestingly, *Nrtn* is expressed in developing and mature sweat glands, the target tissue of cholinergic sympathetic neurons in the mouse (Golden et al., 1999).

In newborn rats and mice *Ret* is expressed in a subpopulation of chromaffin cells in the adrenal medulla (Forander et al., 2001). In the adult rat, adrenal chromaffin cells also express high levels of *Gfra2*, but no *Ret* (Schober et al. 2000). The sympathetic ganglion cells in the adrenal medulla express both *Gfra1* and *Ret* (Schober et al., 2000; Forander et al., 2001; Lindahl et al., 2001). However, Ret or GFRα1 protein expression was not detected in adult rat adrenal medulla (Schober et al., 1999). *Gfra1* and *Gfra2* without *Ret* expression are detected in the nerve fibres of the adrenal cortex of the adult rat (Schober et al., 2000). In newborn mice, the correctly spliced form of *Gfra4* mRNA is expressed in low levels by chromaffin cells, whereas in adults *Gfra4* transcripts are evenly expressed in all cells of the adrenal medulla (Lindahl et al., 2000).

Of the ligands, *Gdnf*, *Nrtn* and *Pspn* are expressed in the rodent adrenal gland (Krieglstein et al., 1996; Xian et al., 1999; Lindahl et al., 2001) and GDNF protein expression has been detected in rat adrenal chromaffin cells (Krieglstein et al., 1998;Schober et al., 1999). The function of GDNF in the adrenal medulla may be to offer trophic support for sensory neurons that innervate the chromaffin cells (Krieglstein et al., 1996), while the function of PSPN or NRTN in the adrenal gland is unknown.

Parasympathetic nervous system

Parasympathetic preganglionic axons arise from neurons in the brainstem and sacral spinal cord. The preganglionic neurons project to postganglionic neurons in parasympathetic ganglia that are situated close to or within the visceral targets. Parasympathetic preganglionic nuclei in the brainstem include the Edinger-Westphal nucleus, the salivatory nuclei, the dorsal vagal nucleus and the nucleus ambiguous. Preganglionic axons from the Edinger-Westphal nucleus project to the ciliary ganglion via cranial nerves, and the ciliary ganglion in turn innervates the constrictor muscles of the eye. The salivatory nuclei also project through cranial nerves to postganglionic neurons in the sphenopalatine, submandibular and otic ganglia, which innervate lacrimal, submandibular and sublingual and parotid glands, respectively. Parasympathetic stimulation induces lacrimal fluid secretion, which moistens the ocular surface, and evokes most of the watery secretion from the salivary glands. The dorsal vagal nucleus innervates thoracic and abdominal targets, such as the lungs, liver, gall bladder, pancreas and the upper intestinal tract, via the vagus nerve, whereas the nucleus ambiguous innervates cardiac ganglia. Parasympathetic preganglionic cell axons in the sacral spinal cord leave the spinal cord via ventral roots and project via the pelvic nerve to parasympathetic postganglionic neurons in the pelvic ganglion plexus. The pelvic plexus innervates the descending colon, bladder and external genitalia (Dodd and Role, 1991).

Neurons of the intrapancreatic ganglia develop from the vagal neural crest cells, first colonizing the foregut (in rats at E11) and then entering the developing pancreas that evaginates from the foregut endoderm to form the dorsal and ventral pancreatic buds (in mice at E9.5-E10.5) (Schwitzgebel, 2001; Kim and MacDonald, 2002). Between days E12 and E13, there is a secondary migration of a subset of crest-derived cells from the duodenum or of a later arriving population of neural crest cells into the pancreatic rudiments (Fig. 5). These crest-derived cells aggregate to form a network of intrapancreatic ganglia, which can be considered as an extension of the enteric nervous system (Kirchgessner et al., 1992).

The pancreas is a compound gland consisting of both endocrine and exocrine tissues, which have specific functions. The endocrine part is organized as islets of Langerhans that secrete hormones crucial for balancing blood sugar levels, namely insulin, glucagon, somatostatin and pancreatic polypeptide. The exocrine tissue produces pancreatic juice that contains bicarbonate and many proteases, lipases and carbohydrases essential for digestion. Pancreatic juice is released through the pancreatic duct into the duodenum, where it neutralizes intestinal gastric acid (Schwitzgebel, 2001; Kim and MacDonald, 2002). The pancreas is richly innervated by sympathetic, parasympathetic and sensory branches. Parasympathetic cholinergic innervation controls the secretion of islet hormones (including insulin) (Brunicardi et al., 1995; Ahren, 2000) as well as the food-induced secretion of pancreatic enzymes (Owyang, 1996; Rogers et al., 1996). The parasympathetic fibres originate from the intrapancreatic ganglia, which receive preganglionic input from the brainstem via the vagus nerve but also direct input from myenteric ganglia (Kirchgessner and Gershon, 1990).

Gdnf mRNA is expressed within or around the migrating parasympathetic precursors and in parasympathetic tissues, but its expression decreases during embryonic development (Golden et al., 1999; Enomoto et al., 2000). *Nrtn*, however, is expressed from E14 onwards in parasympathetic target tissues, such as salivary and lacrimal glands and the pancreas (Golden et al., 1999), and is upregulated postnatally in salivary and lacrimal glands (Enomoto et al., 2000). Parasympathetic precursors express *Ret* and *Gfra1* (Golden et al., 1999; Rossi et al., 2000), but before birth *Gfra1* mRNA is downregulated in many cranial parasympathetic ganglia, including otic, sphenopalatine, ciliary and submandibular ganglia. The *Ret* expression remains postnatally and in addition the expression of *Gfra2* is upregulated in parasympathetic neurons (Enomoto et al., 2000; Rossi et al., 2000).

Enteric nervous system

The enteric nervous system acts to some degree independently of the rest of the autonomic system, although the gastrointestinal tract also receives input from the sympathetic and parasympathetic branches. The network of sensory and motor neurons and glia interconnects the different parts of the digestive tract and regulates intestinal motility, secretion and transfer of substances across the gut epithelium (Grundy and Schemann, 2005). The enteric nervous system innervates not only the digestive tract but also the accessory digestive organs such as the pancreas and the gall bladder. Nerve fibre bundles connect enteric ganglia together to form two plexuses along the intestine, the myenteric plexus and the submucosal plexus. Enteric motoneurons reside in the myenteric plexus and innervate both longitudinal and circular muscle layers. While the myenteric plexus is present along the entire length

of the intestine, the submucosal plexus is present only in the small and large intestines. The myenteric plexus or primary plexus consists of myenteric ganglia and connective internodal strands. Secondary strands arise from the primary plexus, run parallel to the circular muscle and give rise to a deep muscular plexus (dmp) between the circular muscle layer and the submucosal plexus (Wilson et al., 1987). The tertiary plexus forms a network of thin-calibre fibre bundles in the space between the meshes of primary plexus. The tertiary plexus is suggested to be the major site for neurotransmission to the longitudinal muscles in the guinea pig (Llewellyn-Smith et al., 1993).

Acetylcholine is the primary transmitter of excitatory motoneurons, but also peptide transmitters, such as substance P, are released. Inhibitory motoneurons use NO as their primary transmitter instead and such neuropeptides as VIP and neuropeptide Y (NPY) as co-transmitters (Sang et al., 1997). Peristalsis, the intestinal motility that propels ingested material, is mediated by a complex pattern of neural reflexes involving various transmitters that aim to contract the intestinal muscles. The muscle constriction proceeds along the length of the alimentary canal by a simultaneous contraction of the longitudinal muscle and relaxation of the circular muscle pushing the contents of the intestine in the direction of the peristaltic wave. Intestinal neuromuscular transmission is regulated by interstitial cells of Cajal (ICC), which form a network that connects myenteric neurons to smooth muscle cells (Lecci et al., 2002).

Enteric precursors that arise from the vagal neural crest migrate from the hindbrain region in a rostro-caudal wave along the entire length of the developing alimentary tract (Fig. 5). However, most of the enteric neurons in the oesophagus are not vagal derived, instead being populated by trunk-level neural crest cells. An additional source of enteric neurons is the sacral neural crest, although these cells only colonize the postumbilical gut and lie principally in the myenteric plexus (reviewed in Newgreen and Young, 2002a). The enteric neural progenitors from the vagal neural crest reach the foregut priordium at E9.5-10 in the mouse, and the entire length of the gut is populated by E14 (Young et al., 1998). During migration, neural crest cells actively proliferate and finally differentiate into a variety of neuronal subtypes required for normal intestinal motility and function. Some molecules involved in development of the enteric nervous system have been established; for example, sonic hedgehog (Shh) and BMPs may regulate the neuronal number and differentiation of neural crest cells in the gut, while endothelin-3 and its receptor (endothelin receptor B) have a role in the colonization of the distal bowel (Grundy and Schemann, 2005).

Ret mRNA is expressed in presumptive enteric neuroblasts of the vagal crest at E9-11.5 and in the myenteric ganglia of the gut (E13.5-14.5) (Pachnis et al., 1993; Tsuzuki et al., 1995; Durbec et al., 1996), but it is not expressed in sacral-derived cells (Young et al. 1998). Ret immunoreactivity has been detected in developing rat gut from E13.5 onwards and by E15.5 the Ret-immunoreactivity localized to the myenteric plexus (Worley et al., 2000). *Gfra1* is detectable within the gut wall at E14 in both neural crest-derived and mesenchymal cells. Postnatally, *Gfra1* expression weakens and becomes restricted to myenteric and submucosal ganglia. However, *Gfra2* can be detected in newborn enteric nervous system and continues to be expressed strongly in the adult (Gianino et al., 2003).

Gdnf is expressed at high levels in mouse gut muscle layers during embryogenesis. During invasion of the foregut by neural crest progenitors, *Gdnf* is expressed in the mesenchyme of the stomach ahead of the migrating crest cells. As the enteric precursors migrate towards the midgut, *Gdnf* expression rises in a more

posterior region (Natarajan et al., 2002). *Nrtn* mRNA expression begins at E14 in the developing intestinal muscle layers and the mucosa (Golden et al., 1999), but during postnatal development is concentrated in the circular muscle layer (Widenfalk et al., 1997).

Thyroid C-cells

Thyroid glands reside on either side of the trachea and larynx and are connected by a narrow strip of thyroid tissue at the midline. The mature thyroid gland consists of two cell types, the thyroid follicular cells and the parafollicular or C-cells. The C-cells are distributed unevenly within the gland, with most C-cells located in the middle part of the thyroid lobes in small clusters between the follicles. The follicular cells originate from endodermal thickening in the ventral wall of the developing pharynx, which later forms the primitive thyroid bud (Fig. 5) (Santisteban and Bernal, 2005). The C-cell precursors arise from the vagal neural crest and migrate through the fourth pharyngeal arch mesenchyme to the ultimobranchial body epithelium, which is a derivative of the fourth pharyngeal pouch. In the ultimobranchial body, the precursors differentiate into mature calcitonin-producing cells (Fontaine, 1979; Santisteban and Bernal, 2005). The mature thyroid gland forms when the ultimobranchial body fuses with the thyroid rudiment that has migrated caudally to its final position in the trachea. In the mouse, this occurs at E14 (Fig. 5). The parathyroids migrate lateral to the thyroid gland from the dorsal part of the third pharyngeal pouch (Santisteban and Bernal, 2005).

Thyroid and parathyroid glands produce and secrete hormones involved in regulating body metabolism and mineral balance. The thyroid follicular cells synthesize triiodothyronine (T3) and thyroxine (T4), which increase metabolic rate and body growth. Plasma calcium and phosphate levels, on the other hand, are held in homeostasis by the opposing actions of parathyroid hormone (PTH) and calcitonin (CT), which regulate the flux of these minerals between plasma and bone. Two types of cells are responsible for bone renewal: the osteoblasts regulate bone formation and the osteoclasts digest the bone matrix to release calcium. Parathyroid glands secrete PTH, which increases serum calcium concentration by stimulating bone resorption, whereas thyroid C-cells produce and secrete CT hormone, which lowers the plasma calcium concentration towards normal levels by suppressing the activity of the osteoclasts. Specific calcium-sensing G-protein-coupled receptors (CaR) on the C-cell surface detect the rise in calcium concentration (Brown and MacLeod, 2001; Fudge and Kovacs, 2004). The elevated serum calcium levels stimulate the secretion of CT, which then binds to its receptors on bone osteoclasts (Nicholson et al., 1986; Lin et al., 1991). Consequently, activation of different signalling pathways leads to withdrawal of the osteoclasts from the bone surface and reduced production of acid and proteolytic enzymes.

Cell-specific splicing of the CT/CGRPα gene results in production of two distinct peptides, CT exclusively by the thyroid C-cells and calcitonin gene-related peptide-α (CGRPα) throughout the central and peripheral nervous system (Rosenfeld et al., 1983). Although calcitonin has a potent plasma calcium-lowering effect and is successfully used to treat conditions associated with increased osteoclast activity, such as osteoporosis and Paget's disease of bone (Civitelli et al., 1988; Zaidi et al., 2002), its physiological role in bone formation in mammals remains obscure. Not

least because removal of the thyroid gland has little effect on long-term calcium or bone metabolism and because high serum calcitonin levels in medullary thyroid carcinoma do not cause clear osteopetrosis (Zaidi et al., 2002). Surprisingly, mice lacking either the CT/CGRPα or CT receptor gene have increased bone mass due to a higher bone formation rate and display enhanced responsiveness to exogenous PTH (Hoff et al., 2002; Dacquin et al., 2004), suggesting a function for CT in the regulation of bone metabolism.

Like the other neural crest-derived endocrine cell population, adrenal chromaffin cells, the C-cells share biochemical and morphological characteristics with peripheral neurons. C-cells can synthesize serotonin and serotonin-binding protein similarly to enteric neurons (Nunez and Gershon, 1972; Barasch et al., 1987b) and are able to extend neurites in response to NGF in culture (Barasch et al., 1987a; Clark et al., 1995). The C-cell precursors migrate together with enteric precursors from the vagal neural crest, and this has led to the hypothesis that C-cells and enteric neurons share a common origin, resembling that of chromaffin cells and sympathetic neurons of the SA lineage (Barasch et al., 1987a; Anderson, 1993). Furthermore, Mash1, the transcription factor common to autonomic neuronal precursors and chromaffin cells, is also required for thyroid C-cell development in rodents (Clark et al., 1995; Lanigan et al., 1998).

Ret mRNA is detected in the posterior pharyngeal arches and in the ultimobranchial body (Pachnis et al., 1993; Lindahl et al., 2000) and continues to be expressed in adult C-cells, albeit at lower levels (Tsuzuki et al., 1995; Belluardo et al., 1999; Lindahl et al., 2000). In rat, Ret immunoreactivity is detected in juvenile to adult thyroid C-cells (Tsuzuki et al. 1995). *Gfra1* transcripts are seen in thyroid follicular cells in rat (Belluardo et al., 1999), and *Gfra2* mRNA was found in developing mouse parathyroids from E12-E18 (Golden et al., 1999). A low level of *Gfra3* expression was detected in what appeared to be thyroid C-cells (Lindahl et al., 2000). *Gfra4* was observed in the mouse ultimobranchial body together with *Ret* at E12-E16 (Lindahl et al., 2000), and the transcripts encoding the functional GPI-linked form of GFRα4 were expressed in the 3-week-old mouse thyroid gland in a pattern resembling the C-cell distribution, as well as in the parathyroid gland (Lindahl et al., 2000). *RET* and *GFRA4* are co-expressed also in human malignant C-cells such as in medullary thyroid carcinoma (Lindahl et al., 2000). Of the GFLs, *Artn* is seen in the thyroid gland by Northern blot (Baloh et al., 1998b), and moderate levels of *Nrtn* mRNA are detected in epithelial cells of thyroid follicles in rats (Xian et al., 1999).

In vivo functions of GDNF family factors

Genetically engineered mouse models have provided valuable information on the physiological significance of GFLs and their receptors. These studies have revealed functions for GFLs, mostly in the PNS and in kidney development since only minor defects have been reported in the CNS of mice lacking GFLs or their receptor components. Gene ablation studies imply a specific pairing of each GFL and corresponding GFRα *in vivo*. Moreover, it appears that virtually all cells and tissues affected in GFL and GFRα-deficient mice also express Ret, indicating that Ret is the main signalling receptor of GFLs *in vivo*. Mutations in GDNF family ligand and receptor genes, especially in *RET*, have been found in some human diseases.

GDNF/GFRα1/Ret signalling in the development of the enteric and parasympathetic nervous systems

Ret- (Schuchardt et al., 1994), GDNF- (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) and GFRα1- (Cacalano et al., 1998; Enomoto et al., 1998) deficient mice die soon after birth and share a similar phenotype of absent kidneys (see below) and defects throughout the enteric and parasympathetic nervous systems. The enteric neurons of the myenteric plexus of the small and large intestine are completely missing in these mice, resembling HSCR in humans (Parisi and Kapur, 2000). GDNF signalling may be essential not only for the development of the structure but also for proper functioning of the enteric nervous system, as heterozygous *Gdnf*+/-, *Gfra1*+/- and *Ret*+/- mice all have markedly reduced intestinal contractility and neurotransmitter release *in vitro* (Gianino et al., 2003). Different Ret isoforms appear to have distinct functions *in vivo*, as shown by experiments using knock-in mice expressing either RET9 or RET51 (de Graaff et al., 2001). The RET9 homozygous knock-in mice exhibit aganglionosis of the distal colon similar to HSCR and a slightly milder kidney phenotype than the Ret-deficient mice (de Graaff et al., 2001). However, in RET51 knock-in mice, both the enteric nervous system and the kidneys develop normally. The function of GDNF in enteric nervous system development has been conserved evolutionarily, as blocking GDNF signalling in zebrafish prevents migration of enteric neuron precursors. The fish gdnf receptors: *gfra1a*, *gfra1b* and *ret*, are expressed in developing enteric nervous system precursors upon these cells entering the gut. Furthermore, the enteric neurons can be eliminated in zebrafish by injecting antisense oligonucleotides against *ret* or the *gfra1* orthologues (Shepherd et al., 2004). In mammals also at least some parasympathetic neurons require GDNF signalling during their early development since certain cranial parasympathetic ganglia, namely otic and sphenopalatine ganglia, are missing in GDNF-, GFRα1- and Ret-KO mice (Marcos and Pachnis, 1996; Enomoto et al., 2000; Rossi et al., 2000).

GDNF is able to support many central neuronal populations *in vitro*, including motoneurons and midbrain dopaminergic neurons, and appears to promote the survival and axonal branching of motoneurons also *in vivo*. A small but significant reduction in the number of motoneurons was observed in GDNF- and GFRα1-deficient mouse embryos (Moore et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998; Oppenheim et al., 2000). This motoneuron death could be prevented by overexpressing GDNF in muscle and by exposing knockout mouse embryos to exogenous GDNF *in utero* (Oppenheim et al., 2000). Conversely, transgenic mice overexpressing GDNF in muscle show a greater number of motoneurons and hyperinnervation of neuromuscular junctions (Nguyen et al., 1998), suggesting that GDNF-mediated Ret signalling is vital for axonal branching and synapse formation. Furthermore, GDNF/Ret signalling may be important postnatally for synaptic transmission at the neuromuscular junction (Ribchester et al., 1998).

Although GDNF supports the survival of embryonic and adult midbrain dopaminergic neurons, these neurons develop normally in GDNF-, GFRα1- and Ret-KO mice (Marcos and Pachnis, 1996; Moore et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). However, these mice die before the dopaminergic system fully matures. Thus, GDNF may support the survival of dopamine cells postnatally in normal or pathological states (Granholm et al., 2000; Oo et al., 2003), or it may regulate the production or release of dopamine (Bourque and Trudeau, 2000; Yang et al., 2001; Grondin et al., 2003). In addition, GDNF is

proposed to have other functions in the adult brain, such as promoting learning behaviour (Gerlai et al., 2001) and reducing biochemical and behavioural changes associated with drug addiction (Messer et al., 2000; Airavaara et al., 2006).

Somatosensory neurons become dependent on GDNF only after birth *in vitro* (Baudet et al., 2000). The number of sensory neurons is consistently unaffected in the DRG or trigeminal ganglia of late embryonic (E18.5) GDNF-deficient (Oppenheim et al., 2000) or newborn GFRα1-deficient mice (Cacalano et al., 1998; Enomoto et al., 1998). Postnatally, GDNF may be required for target innervation of at least some of the Ret-expressing sensory neurons, as heterozygous *Gdnf*+/- mice were shown to lose myelinated mechanoreceptors in whisker follicles (Fundin et al., 1999). GDNF may also be involved in regulating plastic changes after nerve injury since exogenous GDNF could prevent and reverse sensory abnormalities in a neuropathic pain model in rats (Boucher et al., 2000). Furthermore, GDNF was able to induce regeneration of sensory axons back to the spinal cord, and consequently, to rescue sensory function after dorsal root injury (Ramer et al., 2000). However, some visceral sensory neurons seem to require GDNF for survival because petrosal ganglion neurons are reduced by 40% in newborn GDNF-deficient mice (Moore et al., 1996; Erickson et al., 2001). The chemoafferent sensory neurons of the petrosal ganglion innervate the carotid body, an organ involved in breathing control. Thus, newborn mice lacking *Ret* or *Gdnf* have breathing defects unrelated to the lack of kidneys or impaired enteric innervation (Burton et al., 1997; Erickson et al., 2001). Moreover, mutations in *Ret* gene have been associated with congenital hypoventilation syndrome (CCHS or Ondine's curse) (Fitze et al., 2003).

Despite the generally very similar phenotypes, some differences between the Ret-, GFRα1- and GDNF-deficient mice exist. GDNF-KO mice have a 30% reduction of SCG neurons (Moore et al., 1996), while mice lacking GFRα1 have normal numbers of sympathetic neurons (Cacalano et al., 1998; Enomoto et al., 1998). Ret-KO mice, on the other hand, have deficits in migration and axonal outgrowth of neurons throughout the entire sympathetic chain (Durbec et al., 1996; Enomoto et al., 2001) and also show loss of the cholinergic population of sympathetic neurons in the stellate ganglion (Burau et al., 2004).

GDNF/GFRα1/Ret signalling outside the nervous system

In homozygous GDNF-, GFRα1- and Ret-deficient mice, the ureteric bud fails to develop, or if it develops it does not branch, and kidneys are absent or remain dysplastic and non-functional (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998). Ret-KO mice exhibit mild deficits also in neural crest-derived endocrine cells. They have moderate thyroid C-cell loss (Lindahl et al., 2000) and the adrenal chromaffin cells, although largely normal in morphology, produce reduced levels of adrenalin (Allmendinger et al., 2003). Fascinatingly, mouse models have implied that GDNF/GFRα1/RET signalling could serve as a potential target for designing male contraceptives and in treatment of hair growth disorders. A role for GDNF in the mouse testis was discovered in a study using transgenic loss-of-function and overexpressing models of GDNF. The dosage of GDNF produced by Sertoli cells seems to regulate the cell fate decision of undifferentiated spermatogonial cells (Meng et al., 2000). Hair growth was enhanced in a transgenic mouse line overexpressing Ret in the skin (Kato et al., 2001), and

moreover, heterozygous GFRα1-deficient mice exhibited an accelerated regression period in the hair follicle cycle (Botchkareva et al., 2000).

NRTN/GFRα2 signalling in the development of the parasympathetic and enteric neurons

Mice lacking NRTN (Heuckeroth et al., 1999) or GFRα2 (Rossi et al., 1999) are viable and fertile with no gross deficits in major organs. They share similar impairments in the cholinergic enteric and parasympathetic nervous systems (see Table 3). NRTN- and GFRα2-KO mice have defects of varying degree in the cholinergic parasympathetic innervation of lacrimal and salivary glands. The submandibular ganglion innervating the submandibular and sublingual salivary glands has a reduced number of parasympathetic neurons in both NRTN- and GFRα2-KO mice (Heuckeroth et al., 1999; Rossi et al., 1999). However, in cranial parasympathetic otic and sphenopalatine ganglia, which innervate the parotid gland and the lacrimal gland, respectively, the neurons are present but reduced in size (Rossi et al., 2000). Furthermore, as a result of the reduced lacrimal gland innervation, both NRTN- and GFRα2-deficient mice exhibit diminished tear production and, consequently, tend to keep their eyes closed (pseudoptosis) (Heuckeroth et al., 1999; Rossi et al., 1999). In addition, the parasympathetic nerve fibres that innervate pancreatic islets of Langerhans are reduced in GFRα2-KO mice, leading to impaired islet hormone secretion (Rossi et al., 2005). The sacral system supplying the pelvic organs with parasympathetic innervation seems to require NRTN/GFRα2/Ret signalling as well. Cholinergic parasympathetic innervation of the penis is reduced in GFRα2-deficient mice (Laurikainen et al., 2000), and innervation of the epithelium of reproductive organs is impaired in both GFRα2- and NRTN-deficient mice (Wanigasekara et al., 2004). The cell size of parasympathetic neurons innervating the bladder and the vas deferens is also smaller in these mice (Wanigasekara et al., 2004). Furthermore, the cholinergic innervation of the heart and the volume of the cardiac ganglia are reduced in GFRα2-KO mice (Hiltunen et al., 2000).

While myenteric ganglion cell number is only slightly decreased, the density of the cholinergic, substance P-containing myenteric plexus is reduced in the small intestine of both GFRα2- and NRTN-deficient mice, especially in the duodenum. Moreover, the motility of the gut is impaired *in vitro* (Heuckeroth et al., 1999; Rossi et al., 1999), and interestingly, the release of the excitatory neurotransmitter substance P from the NRTN-deficient mouse colon is also reduced *in vitro* (Heuckeroth et al., 1999). This implies that besides supporting enteric innervation, NRTN/GFRα2-mediated signalling may regulate neurotransmitter release.

As indicated by the reduction of neuronal number and size in many parasympathetic ganglia and in the enteric neural plexus, NRTN/GFRα2-mediated signalling appears to be important for target innervation and maintenance of cell size (Heuckeroth et al., 1999; Rossi et al., 1999, 2000; Wanigasekara et al., 2004). In fact, a switch from GDNF to NRTN dependency is proposed to occur in parasympathetic and enteric neurons during late embryonic development: the early neural precursors are thought to require GDNF/GFRα1 for migration and proliferation, with NRTN/GFRα2 signalling being needed later for the development and maintenance of parasympathetic and enteric target innervation (Rossi et al., 2000; Airaksinen and Saarma, 2002). Although GFRα2- and NRTN-deficient mice display strikingly similar

phenotypes, a clear difference exists. GFRα2-deficient mice show retarded growth after weaning, whereas NRTN-deficient mice grow normally (Heuckeroth et al., 1999; Rossi et al., 1999). The difference between GFRα2- and NRTN-deficient mice in growth, even on the same diet and bred to the same background, suggests that GFRα2/Ret may be activated by another ligand *in vivo* (Wanigasekara et al., 2004). The cause of this growth retardation in GFRα2-KO mice is discussed in the Results and Discussion section of this thesis.

A physiological role for NRTN/GFRα2/Ret signalling in the sensory nervous system has also been proposed. NRTN-deficient mice exhibit a loss of *Gfra2* expressing sensory neurons in DRG and trigeminal ganglion (Heuckeroth et al., 1999), but in GFRα2-deficient mice no loss of sensory neurons has been reported (Rossi et al., 1999; Stucky et al., 2002). However, the diameter but not the number of both unmyelinated and myelinated axons in the saphenous nerve was smaller in GFRα2-KO mice. Furthermore, electrophysiological studies using acutely isolated DRG neuron cultures and skin-nerve preparations from GFRα2-deficient mice indicated that GFRα2-mediated Ret signalling regulates noxious heat transduction of the IB_4-binding subpopulation of sensory neurons (Stucky et al., 2002). The *in vivo* role of GFRα2 in somatosensory neuron function is discussed in the Results and Discussion section of this thesis. Although GFRα2-KO mice have no gross deficits in CNS structures, GFRα2-mediated signalling appears to have a role in brain functions. The response to epileptic stimulus in the hippocampal kindling model was suppressed in GFRα2-deficient mice (Nanobashvili et al., 2000), and they also showed impaired cognitive abilities in common learning and memory tests (Voikar et al., 2004).

GFRα3/ARTN signalling in the migration and initial axon outgrowth of sympathetic neurons

ARTN- and GFRα3-KO mice are viable and fertile and show no gross deficits in major organs (Nishino et al., 1999; Honma et al., 2002). GFRα3-deficient mice also have ptosis, but the mechanism is different from that in mice lacking *Gfra2* or *Nrtn*. The superior cervical ganglion that innervates the lid elevator muscle is smaller and incorrectly located, and consequently, the sympathetic innervation to the lid muscle is reduced (Nishino et al., 1999). In fact, ARTN- and GFRα3-deficient mice have abnormalities in the migration and axonal projection pattern of the entire sympathetic nervous system. The incorrectly positioned sympathetic neuroblasts are unable to obtain neurotrophic support and thus to innervate the target tissues, and eventually, some of the sympathetic neurons die (Nishino et al., 1999; Honma et al., 2002). ARTN is expressed along the migratory route of the sympathetic neuroblasts in smooth muscle cells of blood vessels and may serve as a chemoattractant for the sympathetic neuroblasts. Consequently, ARTN/GFRα3/Ret signalling is needed for the migration and initial axonal outgrowth of developing sympathetic neurons. Although GFRα3 is expressed in nociceptive sensory neurons of DRG and trigeminal ganglia (Orozco et al., 2001), no deficits in sensory neurons have been found in either ARTN- or GFRα3-KO mice.

PSPN may protect the brain from ischaemia

PSPN-deficient mice are viable and fertile, with no gross deficits in any tissues examined, including the CNS and the thyroid gland (Tomac et al., 2002). Even extensive behavioural tests showed no prominent differences between PSPN-KO mice and their wild-type littermates. However, in experimentally induced focal cerebral ischaemia, PSPN-deficient mice showed an increased sensitivity to stroke (Tomac et al., 2002). Pretreatment of the KO mice with low doses of PSPN before middle cerebral artery occlusion markedly reduced the infarction size and enhanced vertical locomotor activity, while high doses of PSPN increased the infarction size. Low PSPN doses also protected cortical neurons from hypoxia-induced cell death *in vitro* (Tomac et al., 2002). The phenotype of GFRα4-deficient mice is described in the Results and Discussion section of this thesis.

RET mutations in human diseases

Mutations in RET have been reported in several neural crest disorders. Activating mutations of RET are found in the majority of families with multiple endocrine neoplasia type 2A and 2B (MEN2A and MEN2B) cancer syndromes and familial medullary thyroid carcinoma (FMTC). MEN2A and MEN2B manifest as medullary thyroid carcinoma (MTC) originating from the thyroid C-cells and pheochromocytoma, which arises from chromaffin cells of the adrenal medulla. In addition, hyperparathyroidism develops in some MEN2A patients. MEN2B patients show a more complex phenotype, including skeletal abnormalities and enteric and mucosal neuromas. FMTC is characterized by MTC as its only disease phenotype. RET mutations are found also in sporadic MTC, but in sporadic tumours arising from the adrenal or parathyroid glands, RET mutations are rare. In addition, somatic rearrangements of RET are involved in papillary thyroid carcinomas (PTC) arising from thyroid follicular cells. In MEN2A and FMTC, cysteine substitutions (notably in exons 10 and 11) in the extracellular domain induce a ligand-independent dimerization and constitutive activation of RET. Conversely, in MEN2B, a single substitution of a methionine residue at position 918 in the tyrosine kinase domain activates RET by changing its substrate specificity (see Fig. 4) (Hansford and Mulligan, 2000; Manie et al., 2001; Takahashi, 2001). Although *Ret* is expressed in various tissues, such as the kidneys and testis (Pachnis et al., 1993; Tsuzuki et al., 1995), the tumours in MEN2 syndromes are restricted to just a few tissues. GFRα receptors have been proposed as modifiers that could restrict the occurrence of tumours to specific tissues. *GFRA4* is expressed in thyroid C-cells and in MTC, indicating that GFRα4 might be necessary for development of C-cell hyperplasia or MTC (Lindahl et al., 2001; Vanhorne et al., 2005). Alternatively, other GFRα receptors could interfere with the dimerization of mutated RET, thus inhibiting tumour formation in tissues unaffected in MEN2 (Kawai et al., 2000). However, some FMTC patients have been reported to lack a kidney (Lore et al., 2000) resembling the phenotype of Ret-deficient mice.

Inactivating mutations of *RET* have been reported in about 50% of patients with inherited HSCR, and in 10-20% of sporadic cases of HSCR. HSCR is a developmental deficit characterized by lack of innervation along variable lengths of the hindgut. The loss of colonic ganglia leads to chronic constipation and intestinal

obstruction (Manie et al., 2001; Takahashi, 2001; Newgreen and Young, 2002b). Mutated RET may affect both survival and migration of the enteric precursors and cause HSCR (Bordeaux et al., 2000). Although GDNF and NRTN mutations have been found occasionally in patients with HSCR (Eketjall and Ibanez, 2002), mutations in GFRα receptors have not been reported (Borrego et al., 2003). HSCR is a multifactorial disease modulated by interactions between two or more disease genes, and interactions between Ret and endothelin receptor type B have been suggested (Carrasquillo et al., 2002). Ret-deficient mice exhibit intestinal aganglionosis, but the phenotype of heterozygous GDNF mutant mice more closely resembles human HSCR. These mice show a variable severity of symptoms, ranging from mild hypoganglionosis to segmental aganglionosis (Shen et al., 2002). Rarely, both HSCR and MEN2A or FMTC may occur in the same patient. This phenotype is associated with mutations specifically in cysteine 609, Cys618 or Cys620 that disturb the translocation of RET to the cell membrane (Manie et al., 2001; Takahashi, 2001). Thus, RET is insufficiently available in the developing enteric nervous system, causing apoptosis while at the same time constitutively activated RET causes uncontrolled proliferation of endocrine cells.

AIMS OF THE STUDY

The general aims of this thesis were to study the physiological functions of GFRα4 and the novel aspects of GFRα2 function in the PNS using gene deficient mouse models.

Specific aims were as follows:

- To identify mechanisms contributing to postnatal growth retardation in GFRα2-deficient mice
- To examine the function of GFRα2 in sympathetic cholinergic neurons and their target innervation *in vivo*
- To determine the function of GFRα2 in sensory neurons and their target innervation *in vivo*
- To study the *in vivo* function of GFRα4 by creating GFRα4-deficient mice

MATERIALS AND METHODS

Most of the materials and methods used in this study are described in detail in the original articles (I-IV). The local ethics committee for animal research at the University of Helsinki approved all animal experiments.

The generation of GFRα4-KO mice is described in detail in study IV. Briefly, a fragment of the *Gfra4* gene containing exons 2-5 was replaced with a PGKneo cassette (neomycin resistance gene under the phosphoglycerate promoter) by homologous recombination in R1 embryonic stem cells. Chimeric mice derived from these cells were bred to C57NL/6JolaHsd and 129SvHsd females to establish heterozygotes. The transgenic offspring were genotyped from tail DNA by PCR. In most experiments, hybrids (C57BL/6 x 129Sv) of wild-type and GFRα4-KO littermates obtained by inter-crossing the congenic heterozygous parents were used. In addition, wild-type and GFRα4-KO mice obtained from homozygous matings were used in some of the experiments (IV).

Table 1. List of probes used for *in situ* hybridization.

Probe	Species	Size	Nucleotides	Vector
Gfra1	Mouse	777 bp	1-777	pPT7T3
Gfra2	Rat	2002 bp	1-2002	pBS
Gfra3	Mouse	1193 bp	95-1288	pPCDNA3
Gfra4 3' EST	Mouse	497 bp	1-497	pPT7T3
Gfra4 5' exon1a	Mouse	127 bp	1-5, 122 bp 5'UTR	pPCRII
Ret	Mouse	646 bp	2534-3217	pPBS
Nrtn	Mouse	587 bp	349-936	pCDNA

The methods for *in situ* hybridization and RT-PCR for *Gfra* mRNA analyses are described in the original articles and references therein (I, II, IV). The probes used are listed in Table 1. The procedures for immunohistochemical detection and for histological stainings are described in detail in the original articles (I-IV). The antibodies used are provided in Table 2. The quantification of nerve fibre density is described in studies II and III. Cell counts were performed as decribed in studies II, III and IV. Cell size distribution analyses are outlined in studies II and III. Co-localization of different markers using confocal microscopy was used in studies I, III and IV.

Sensitivity to painful stimuli was evaluated using standard behavioural tests. More detailed description of hot plate, tail withdrawal, temperature choice, formalin and von Frey test for mechanical sensitivity are found in study III and the references therein. Chronic inflammatory hyperalgesia was evaluated from mice anaesthetized briefly with isofluran and injected with 30 µl of 50% complete Freund's adjuvant (Sigma) under the plantar surface of the left hind paw. Before and 24 and 72 h after

the injection, the thermal nociceptive threshold was determined according to the method described in Hargreaves et al. (1988). A radiant heat source (Basile plantar test, Ugo Basile) located under the floor was targeted at the medial plantar surface of the hind paw, and the latency to withdraw from the stimulus was measured. The withdrawal latency was determined four times from both the injured and the uninjured hind paw at 5-min intervals, with results being expressed as percentage hypersensitivity of the injected paw compared with the uninjected paw (unpublished data).

Table 2. List of primary antibodies and lectin used in immunohistochemistry.

Antibody/ Lectin	Host	Source	Dilution	Study
Calcitonin	Rabbit polyclonal	DAKO	1:500	IV
Calcitonin	Goat polyclonal sc-7784	SantaCruz	1:1000	IV
CGRP	Rabbit polyclonal AB5920	Chemicon	1:1000	III
GFRα2	Goat polyclonal AF429	R&D	1:200	I, II, III
GFRα4	Goat polyclonal AF1677	R&D	1:400	IV
Biotin-IB_4 lectin	*Bandeiraea simplifocia*	Sigma	1:100	III
nNOS	Rabbit polyclonal AB5380	Chemicon	1:500	I
Peripherin	Rabbit polyclonal AB1530	Chemicon	1:500	III
PGP9.5	Rabbit polyclonal AB1761	Chemicon	1:400	I, II, III
PGP9.5	Sheep polyclonal AHP508T	Serotec	1:50	III
P2X3	Guinea pig polyclonal AB5896	Chemicon	1:500	III
PNMT	Rabbit polyclonal 22572	DiaSorin	1:1000	unpublished data
Ret	Goat polyclonal AF482	R&D	1:20	IV
S100β	Rabbit polyclonal AF482	Swant	1:500	I
Substance P	Rat monoclonal NC1	Medicrop	1:200	I
TH	Rabbit polyclonal AB152	Chemicon	1:200	II
TH	Sheep polyclonal AB1542	Chemicon	1:300	I, data unpublished
TRPV1	Rabbit polyclonal	(Tominaga et al., 1998)	1:2000	III
VAChT	Goat polyclonal AB1578	Chemicon	1:800	I, II
VIP	Rabbit polyclonal 11428	Progen	1:100	II

Thyroid CT hormone levels were measured by immunoradiometric assay (Rat calcitonin IRMA kit, Immutopics, San Clemente, CA, USA) (IV). Bone formation rate was estimated using *in vivo* double calcein fluorescent labelling (Parfitt et al., 1987; Hoff et al., 2002) (IV). Adrenal gland catecholamine levels were measured using high performance liquid chromatography (HPLC). The dissected adrenals were homogenized in 100 µl/10 mg 0.4 M percloric acid. The homogenate was centrifuged and the supernatant was injected into the HPLC system with a CMA/200 autoinjector (CMA Microdialysis). The system for determining noradrenaline and adrenaline levels consisted of an ESA Coularray^{TM} electrochemical detector equipped with a model 5014b microdialysis cell and two analytical cells (ESA Inc.) and an ESA HPLC pump. The column (Spherisorb ODS2, Waters) was kept at 40ºC with a column heater (Croco-Cil, Clouzaeu, InfoLabo). The flow rate of the mobile phase (0.1 M $NaOH_2PO_4$ buffer, pH 4.0, containing 1 mM octane sulphonic acid, 16% methanol and 450 mg/l EDTA) was 1 ml/min.

The method for measuring *in vivo* pancreatic secretion and the charcoal transit test for gut motility are described in study I. Food and water intake, motor activity, basal metabolic rate, wet-mash feeding and faecal-fat analysis were performed as described in study I.

RESULTS AND DISCUSSION

A. GFRα2 in peripheral neurons

The development of intrapancreatic ganglia requires GFRα2 (I)

NRTN signalling via GFRα2 is required for parasympathetic innervation of salivary and lacrimal glands (Enomoto et al., 2000; Rossi et al., 2000), suggesting that GFRα2-mediated signalling might be necessary for the parasympathetic innervation of also other exocrine tissues, such as the pancreas. Expression of *Nrtn* mRNA increases during the late embryonic development of the mouse pancreas (Golden et al., 1999). In adult mice the GFRα2 protein was expressed together with several neuronal markers, including nitric oxide synthase (NOS), VIP and protein gene product 9.5 (PGP9.5), in the intrapancreatic ganglia. In addition, S100β-positive satellite cells surrounding the neuronal cell bodies in intrapancreatic ganglia and the terminal Schwann cells surrounding the axons in the exocrine pancreas expressed GFRα2 (I; Fig. 3). GDNF has been proposed to bind to a secreted form of GFRα1 that is released from the Schwann cell membrane. This GFL/GFRα complex could then activate Ret in the axons. The secreted GFRα receptors might act as directional cues providing positional information for Ret-expressing axons (Ledda et al., 2002). Similarly GFRα2 expressed by glial cells in the exocrine pancreas might act as an outgrowth promoting chemoattractant for parasympathetic GFRα2/Ret-expressing neurons.

Whereas in wild-type mice several neuronal clusters were found in the exocrine pancreas, virtually no intrapancreatic ganglia were detected in the GFRα2-deficient mice. The total number of neuronal profiles was significantly reduced (83%) in adult GFRα2-KO mice (I; Fig. 4). A small proportion (15%) of NOS-positive neurons did not express GFRα2 protein and may represent the population of neurons remaining in KO mice (I; Fig. 3c). At P4 there was already a profound (~85%) reduction in the pancreatic PGP9.5-positive neuronal profiles and an 80% decrease in the number of intrapancreatic ganglia in GFRα2-KO mice. Thus, similarly to submadibular parasympathetic ganglia, intrapancreatic ganglia require NRTN/GFRα2 signalling to achieve the proper number of neurons (Enomoto et al., 2000; Rossi et al., 2000). Pancreatic neuronal progenitors arrive at the pancreatic rudiment between days E12 and E13 after colonizing the foregut (Kirchgessner et al., 1992). Neuronal loss in GFRα2-KO mice appears to occur largely between E15 and birth and is at least partly due to increased cell death in both the intrapancreatic and submandibular ganglia (Lähteenmäki and Airaksinen, unpublished results).

In accord with the reduced number of intrapancreatic neurons, a severely reduced (~90%) density of cholinergic (acetylcholine esterase (AChE), VAChT- and VIP-positive) innervation in the exocrine pancreas of adult GFRα2-deficient mice was observed (I; Fig. 4e,f). AChE staining showed an apparently normal cholinergic innervation in the islets of Langerhans in the GFRα2-deficient mice (I; Fig. 4e,f), but by using the more specific parasympathetic markers VIP and VAChT, the islet innervation was also found to be reduced (Rossi et al., 2005). In contrast to cholinergic parasympathetic innervation, the density of sympathetic innervation of the exocrine (I; Fig. 4g,h) or endocrine (Rossi et al., 2005) pancreas was not significantly different. These results are consistent with previous reports showing reduced or absent

cholinergic parasympathetic innervation of various exocrine glands, but intact noradrenergic sympathetic innervation (see Table 3) (Rossi et al., 1999, 2000).

GFRα2 is needed to maintain the cell size of cholinergic sympathetic neurons (II)

Sympathetic innervation of mouse fore paw sweat glands originates from the stellate ganglion and develops between postnatal days P4 and P20 (Francis and Landis, 1999). In agreement with an earlier report (Golden et al., 1999), *Nrtn* mRNA expression in postnatal mouse sweat glands was detected. The expression was strong in newborn mice, but at P21 hardly exceeded background expression (II; Fig. 1). A subpopulation of sympathetic neurons is known to express *Ret* and *Gfra2* transcripts perinatally (Nishino et al., 1999; Enomoto et al., 2001), but whether this population represents the cells that switch from noradrenergic to cholinergic expression is unknown. In this study GFRα2 protein was detected in a subpopulation of P10 stellate ganglion neurons in mice (II; Fig. 1). However, in adult mice, the expression was hardly detectable. Double-labelling studies with antibody for VIP showed that most if not all of the GFRα2-positive neurons were cholinergic (II; Fig. 1I,J), since VIP is co-expressed with various cholinergic markers (Morales et al., 1995; Asmus et al., 2000). GFRα2 immunoreactivity was also detected in cellular structures that were VIP-negative (II; Fig. 1I,J). These unidentified structures may represent VIP-negative neuronal cell bodies, satellite cells or nerve fibres. Thus, the results suggest that at least part of the postnatal *Gfra2* expression represents the cholinergic population.

Previous studies have found no neuronal losses in the sympathetic SCG or deficits in noradrenergic TH-positive innervation of various tissues in NRTN- or GFRα2-deficient mice (Heuckeroth et al., 1999; Rossi et al., 1999, 2000; Enomoto et al., 2000; Hiltunen et al., 2000), although NRTN is able to support the survival of sympathetic neurons in culture (Kotzbauer et al., 1996). However, expression of GFRα2 in the cholinergic sympathetic neurons and *Nrtn* expression in the sweat glands suggest a function for GFRα2 in the development of sympathetic cholinergic neurons. We examined the relative density and morphology of VIP-positive stellate ganglion neurons between GFRα2-deficient mice and their wild-type littermates. Although the number of VIP-positive neuronal profiles did not differ between the genotypes, their mean area was significantly reduced in GFRα2-KO mice (II; Fig. 2). By contrast, the mean area of TH-positive noradrenergic neuronal profiles was unaltered in the KO mice, and moreover, the estimated volume of the stellate ganglion itself was not significantly different between the genotypes (II). Thus, consistent with previous reports, the number and size of the noradrenergic population of sympathetic neurons are not dependent on GFRα2-mediated signalling.

A subpopulation of sympathetic neurons expresses cholinergic markers already during embryonic development, before peripheral target innervation (Schafer et al., 1997). Since GDNF and NRTN can induce cholinergic differentiation in embryonic chick sympathetic explants (Brodski et al., 2000), GFLs may be required for the early cholinergic differentiation of sympathetic neurons *in vivo*. The number of VAChT- and choline acetyltransferase (ChAT)-positive sympathetic cholinergic neurons is reduced in newborn Ret-deficient mice, suggesting that early cholinergic differentiation of sympathetic neurons requires Ret signalling also in mammals (Burau et al., 2004). However, the normal amount of sympathetic cholinergic neurons in adult GFRα2-KO mice implies that the ligand for early cholinergic differentiation is something other than NRTN. Target tissues of the early cholinergic sympathetic

neurons are unknown (Guidry and Landis, 1998), but the early sympathetic cholinergic differentiation appears to occur independently of cytokines. Therefore, the early cholinergic neurons may represent a population other than the ones innervating sweat glands (Stanke et al., 2000) (see Fig. 6).

GFRα2 is required to maintain the cell size of IB_4-positive sensory neurons (III)

Ret and *Gfra* receptor expression in DRG begins during embryonic development and continues to adulthood (Golden et al., 1999; Baudet et al., 2000), while *Nrtn* mRNA is expressed in embryonic and adult skin epidermis (Luukko et al., 1998; Golden et al., 1999). This suggests a function for NRTN in the development of cutaneous Ret/GFRα2-positive sensory neurons. In a previous report, GFRα2-mediated signalling was proposed to have a function in heat transduction in the IB_4-binding nociceptors (Stucky et al., 2002). In mouse DRG, GFRα2 was localized almost exclusively in the non-peptidergic population of sensory neurons; approximately 80% of the IB_4-binding neurons expressed GFRα2 and 70% of the GFRα2-positive neurons bound IB_4 (III; Fig. 1, Table 1). Most GFRα2-positive neurons (~70%) were unmyelinated given that they co-localized with peripherin, a marker for unmyelinated neurons. The peripherin-negative GFRα2-expressing neurons probably represent thinly myelinated nociceptors since their average size did not differ from the size of the peripherin-positive GFRα2-expressing neurons (III; Fig. 1G-I). Non-peptidergic sensory neurons often express the purinergic receptor $P2X_3$ (Vulchanova et al., 1998). We found GFRα2 expression in 85% of $P2X_3$-positive neurons. By contrast, few (1.5%) GFRα2-positive neurons expressed CGRP, the marker for the peptidergic population (III; Fig. 1, Table 1). In summary, a large majority of small, unmyelinated, non-peptidergic neurons in mouse DRG express GFRα2.

Consistent with normal numbers of unmyelinated axons in cutaneous nerves and IB_4-binding neurons in DRG cultures, acutely isolated from GFRα2-KO mice (Stucky et al., 2002), the density of $P2X_3$-positive neurons in lumbar DRG was similar in wild-type and GFRα2-deficient mice (III; Fig. 2). However, *in vivo* the IB_4-binding and $P2X_3$-positive DRG neuronal profiles were significantly (~30%) smaller in GFRα2-KO mice than in their wild-type littermates (III; Fig. 2C,F), which is compatible with the smaller calibre of unmyelinated nerve fibres in GFRα2-KO mice (Stucky et al., 2002). Accordingly, NRTN-deficient mice have been observed to exhibit reduced size of sensory neurons expressing *Gfra2* (Heuckeroth et al., 1999), but contrary to our data, also the number of *Gfra2*-expressing DRG neurons was reduced in NRTN-KO mice. However, this may be due to downregulation of *Gfra2* expression and not cell death. As expected from the lack of GFRα2 expression in the peptidergic DRG neurons in wild-type mice, the CGRP-positive neuronal profiles in GFRα2-KO mice were of normal size (III; Fig. 2I). Thus, similar to many autonomic cholinergic neurons, GFRα2-mediated signalling (presumably through Ret) is required for cell size but not survival of a subpopulation of cutaneous primary sensory neurons.

B. GFRα2 in peripheral innervation

Reduced innervation of pancreas and small bowel in GFRα2-KO mice (I)

During embryonic development *Nrtn* mRNA is dispersed in both the muscle layers and the mucosa of the intestine (Golden et al., 1999). We found postnatal *Nrtn* expression specifically in the circular muscle layer and GFRα2 protein expression in the myenteric and submucosal nerve plexuses and ganglia of newborn and adult mouse small intestines (I; Fig. 1). GFRα2 co-localized with a marker for excitatory neurons (substance P) in the myenteric ganglia and fibre bundles of the myenteric and submucosal layers of the small intestine. Many enteric glial cells within the ganglia and in muscle and mucosal layers were also GFRα2 immunoreactive (I; Fig. 1i-k). The excitatory AChE-positive fibre density was previously shown to be reduced in the small intestine of GFRα2-deficient mice (Rossi et al., 1999). Results in this study reveal that the number of SP-containing myenteric neuronal cell bodies was also reduced (~35%) in the duodenum of GFRα2-KO mice compared with their wild-type counterparts. A severe reduction in the density of SP-positive nerve fibres was found in the myenteric ganglion cell layer of the small bowel in GFRα2-deficient mice as compared with their littermates (I; Fig. 2). This reduction was most obvious in the tertiary plexus suggesting that the actual fibres, and not only the transmitter, are reduced. By contrast, the density of SP-positive fibres in the dmp layer was not significantly reduced in GFRα2-KO mice (I; Fig. 2c,d). This implies that a different mechanism controls the density of SP-containing fibres in the two layers. A partly overlapping GDNF/GFRα1-mediated Ret signalling might explain the restricted enteric innervation deficit in GFRα2-KO mice, since many enteric neurons (Chalazonitis et al., 1998) express GFRα1 postnatally. The dependence of the tertiary plexus on GFRα2 cannot be explained by lack of GFRα2 expression in dmp-projecting neurons, because GFRα2 is expressed similarly in these two layers. Approximately 25% of SP neurons in myenteric ganglia are GFRα2-negative but whether they project preferentially to the dmp layer is unknown.

GFRα2 is required for sympathetic cholinergic target innervation (II)

The sympathetic innervation of the interdigital sweat glands in fore paw footpads was analysed using several cholinergic markers, namely VIP and VAChT antibodies and AChE histochemistry (II). Quantification of the VIP- and VAChT-positive innervation revealed a significant reduction (~70%) in nerve fibre density in adult GFRα2-deficient mice (II; Fig. 3). Moreover, AChE histochemistry, which is not completely specific for cholinergic fibres revealed a 50% reduction in sweat gland innervation (II; Fig. 3). Similarly to the wild-type mice, the remaining cholinergic fibres in the KO mice lacked catecholamine fluorescence (II, data not shown). Pan-neuronal marker PGP9.5 was used to assess the total innervation density in sweat glands, which was as expected reduced. Approximately 20% of the PGP9.5-positive nerve fibres in the sweat glands of both wild-type and GFRα2-KO mice were VAChT-negative (II, data not shown). These non-cholinergic fibres probably represent sensory fibres (Navarro et al., 1995). In contrast to the loss of the majority of sympathetic cholinergic fibres in the footpad, noradrenergic (TH-positive) sympathetic innervation of blood vessels around sweat glands was intact in GFRα2-

KO mice (II; Fig. 3G, H). The first sympathetic axons are found in the mouse fore paw sweat glands a few days after birth. At that time, the fibres do not express cholinergic markers, but are TH-positive and show catecholamine fluorescence (Guidry and Landis, 1998). During the first postnatal weeks in rodents the sweat gland sympathetic innervation plexus reaches adult density, the gland grows in size, and the neurons acquire cholinergic characteristics (Landis and Keefe, 1983). We found that the density of cholinergic VAChT-positive fibres in the footpads of GFRα2-KO mice at P21 was as much reduced as in the adult. The density of TH-positive innervation around sweat glands, by contrast, appeared similar between the genotypes at P4, indicating that the sympathetic fibres reach the target area successfully, but fail to reach normal density during the first postnatal weeks (II).

In rodents, the only other known postganglionic sympathetic cholinergic target is the periosteum, the connective tissue surrounding the bone (Hohmann et al., 1986; Asmus et al., 2000). Chemical sympathectomy studies suggest that sympathetic periosteal innervation may regulate bone resorption (Hohmann et al., 1986; Cherruau et al., 2003). Tracing studies have shown that VIP-positive nerve fibres around the ribs and sternum originate from the cholinergic neurons in the thoracic sympathetic ganglia. The first sympathetic axons arrive around the periosteum at E17 in the mouse (Asmus et al., 2000). Consistent with this, in present study *Nrtn* transcripts were detected around developing bones, including ribs, at E16.5 and in newborn mice (II; Fig. 4). A sparse network of varicose cholinergic nerve fibres surrounded the ribs and sternum of wild-type mice, but no VIP- or VAChT-positive fibres were found in the periosteum of GFRα2-deficient mice (II; Fig. 4 B, D).

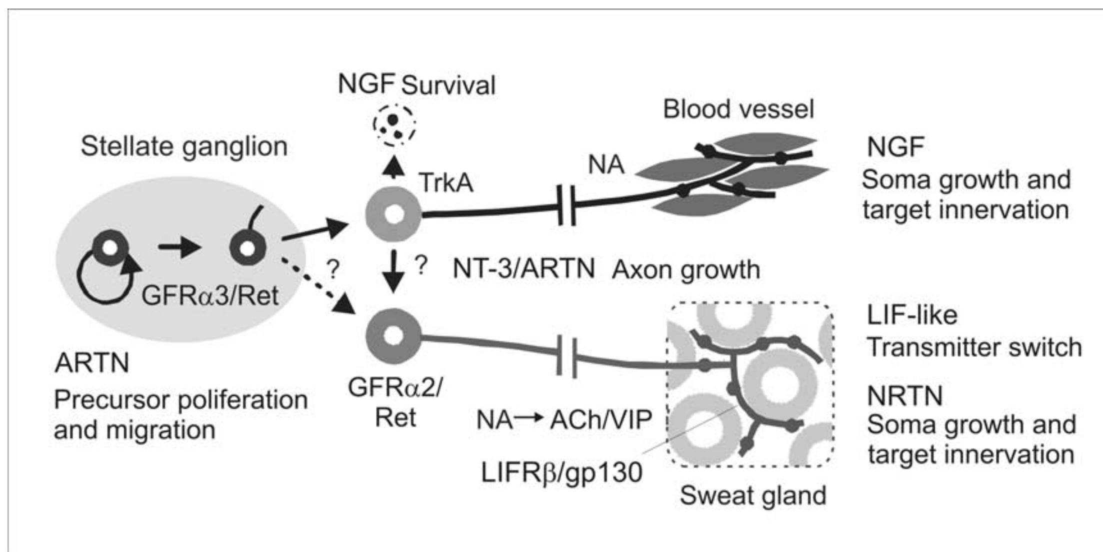

Figure 6. A model for the development of sympathetic cholinergic neurons. Development of sympathetic neurons innervating sweat glands requires simultaneous and sequential actions of a number of factors. ARTN produced by blood vessels along the migratory route of sympathetic neuroblasts is needed for proper migration and proliferation. NGF secreted from the target tissue supports the survival of neurons. Proximal axon extension is promoted by ARTN via GFRα3/Ret and NT-3 via TrkA. Final target innervation of noradrenergic sympathetic neurons requires NGF via TrkA, but cholinergic sympathetic neurons may need NRTN signalling through GFRα2/Ret. The switch from noradrenergic (NA) to cholinergic (ACh/VIP) may require one or several LIF-like factors via LIFRβ/gp130. Adapted from II.

Separate factors appear to promote target innervation and cholinergic differentiation of sympathetic neurons. Sympathetic axons grow successfully into the footpad in mice lacking sweat glands, indicating that the axons do not need any factors produced by the sweat glands to reach the target area (Guidry and Landis, 1995). Furthermore, the normal expression of cholinergic markers in the remaining sympathetic nerve cells and fibres innervating the sweat glands in GFRα2-deficient mice suggests that NRTN does not act as a target-derived "cholinergic differentiation factor" *in vivo* (II; Fig. 2-3). This is in accord with the current hypothesis that the sweat gland-derived cholinergic differentiation factor is an unknown member of the cytokine family that signals through the LIFRβ receptor (Cowen et al., 1996; Ernsberger and Rohrer, 1999; Francis and Landis, 1999).

The present results suggest that NRTN may be a general target-derived innervation or branching and maintenance factor for postganglionic cholinergic axons throughout the autonomic nervous system. Sympathetic cholinergic neurons are able to survive in the absence of target innervation in GFRα2-KO mice (II), which is consistent with the lack of trophic dependence of postnatal sympathetic neurons (Easton et al., 1997). Moreover, sympathetic cholinergic neurons resemble the parasympathetic sphenopalatine ganglion neurons that also survive in the absence of target innervation in GFRα2-KO mice (Rossi et al., 1999). However, the smaller size of the neurons and reduced innervation indicate that NRTN acts as a target-derived trophic factor that helps the sympathetic cholinergic neurons to increase or maintain cell size and innervation as it does for many other autonomic cholinergic neurons (Airaksinen and Saarma, 2002). In contrast to the sympathetic cholinergic population, GFRα2 expression is not required for the target innervation of noradrenergic neurons in any tissues examined, including footpad blood vessels (II; Fig. 3), the heart (Hiltunen et al., 2000) and exocrine glands (Rossi et al., 2000). Sympathetic neurons innervating these tissues depend on neurotrophins and express TrkA, but apparently do not express GFRα2.

The development of the sympathetic cholinergic neurons innervating the sweat glands and possibly the periosteum appears to require successive or simultaneous action of several factors secreted from intermediate and final target tissues (see Fig. 6 for summary). While the molecular mechanism that triggers axon initiation remains unknown, hepatocyte growth factor (HGF) signalling has been proposed (Glebova and Ginty, 2005). ARTN produced by nearby blood vessels may promote proliferation and migration of sympathetic neuroblasts as well as proximal axonal outgrowth (Honma et al., 2002). Another candidate for axon growth is NT-3, which is also expressed in blood vessels (Francis et al., 1999). However, neither NT-3 nor ARTN is able to induce axon initiation. Some neurons may extend axons before migration is complete, implying that soma migration may occur passively, with axons leading the way for migrating cell bodies. Thus, the migration defects seen in ARTN-KO mice may be due to axon growth defects, or conversely, reduced axon extension may lead to improper migration (Glebova and Ginty, 2005). On the other hand, NGF secreted from the target organs promotes the survival of all sympathetic neurons and the final target innervation of many sympathetic noradrenergic neurons (Glebova and Ginty, 2004). Final target innervation may also be under inhibitory control by ligands such as BDNF or proNGF via low affinity neurotrophic receptor p75. However, the final target innervation and axonal branching of the sympathetic cholinergic neurons that occurs simultaneously with the growing target tissue may require NRTN. Thus, the cholinergic sympathetic neurons apparently switch trophic factor classes initially from GFL-Ret signalling to neurotrophin-TrkA signalling, and again back to GFL-Ret

signalling. During the final target innervation phase, the sympathetic neurons switch from a noradrenergic to a cholinergic phenotype, which may require one or more unknown (LIF-like) factors that signal through LIFRβ/gp130 and are secreted by the target tissues (Ernsberger and Rohrer, 1999; Francis and Landis, 1999).

Reduced density of free nerve endings in GFRα2-KO mouse footpad epidermis (III)

Although *in vitro* NRTN is able to support the survival and neurite outgrowth of DRG neurons (Kotzbauer et al., 1996; Rossi et al., 1999; Paveliev et al., 2004), primary sensory neurons do not seem to require GFRα2-mediated signalling for survival or axon growth into the peripheral target area. GFRα2-deficient mice have normal numbers of sensory neurons in trigeminal ganglion (Rossi et al., 1999) and DRG (III) as well as sensory axons in the saphenous nerve (Stucky et al., 2002). However, the nerve fibre density in the epidermis, visualized using antibody against pan-neuronal marker PGP9.5, was clearly reduced (~70%) in GFRα2-KO mice compared to their wild-type littermates. In contrast, the density of CGRP-positive nerve fibres in the epidermis as well as the dermal fibre density appeared similar between the genotypes (III; Fig. 3). Consistent with a previous report (Zylka et al., 2005), we found CGRP expression in ~30% of PGP9.5-positive nerve fibres in wild-type mouse epidermis. In the GFRα2-KO mouse epidermis, by contrast, nearly 60% of the remaining PGP9.5-positive fibres expressed CGRP (III). Since also GDNF is expressed in developing mouse skin (Golden et al., 1999), and *Gfra1* and *Gfra2* are expressed in partly overlapping subpopulations of IB_4-binding neurons (Bennett et al., 1998), the remaining CGRP-negative nerve fibres in GFRα2-KO mouse epidermis may be GDNF-dependent. Interestingly, the morphological changes observed in the cutaneous sensory neurons of GFRα2-deficient mice (Stucky et al., 2002) are the opposite of those found in mice overexpressing GDNF in skin keratinocytes (Zwick et al., 2002). The density of IB_4-binding in inner lamina II of the spinal cord dorsal horn was similar between genotypes, indicating that central target innervation of non-peptidergic neurons is not affected in mice lacking GFRα2 (III; Fig. 4).

Reduced density of epidermal sensory nerve endings is often found in human neuropathic pain syndromes (Chien et al., 2001), including diabetic and idiopathic small fibre neuropathy (Holland et al., 1997) and postherpetic neuralgia (Fields et al., 1998;Oaklander, 2001). The extent of the nerve fibre loss is positively correlated with clinical severity. NRTN/GFRα2-mediated signalling may be altered in these conditions since loss of epidermal innervation is often present with no obvious defect in the peripheral nerve (Periquet et al., 1999;Herrmann et al., 1999). Furthermore, NRTN has been shown to stimulate growth and branching of cutaneous axons in diabetic mice (Christianson et al., 2003), and thus, the GFRα2-signalling pathway may prove to be a target of therapy for sensory regeneration and persistent pain. Peripheral neuropathies usually also involve autonomic and motor components. Selective cholinergic dysautonomia involves deficits in both cholinergic autonomic and small sensory fibres but not in sympathetic adrenergic fibres (Warner et al., 2002). The obvious similarity of this rare disease with the phenotype of GFRα2-deficient mice (II, III) suggests that impaired NRTN/GFRα2 signalling could be involved in its pathogenesis.

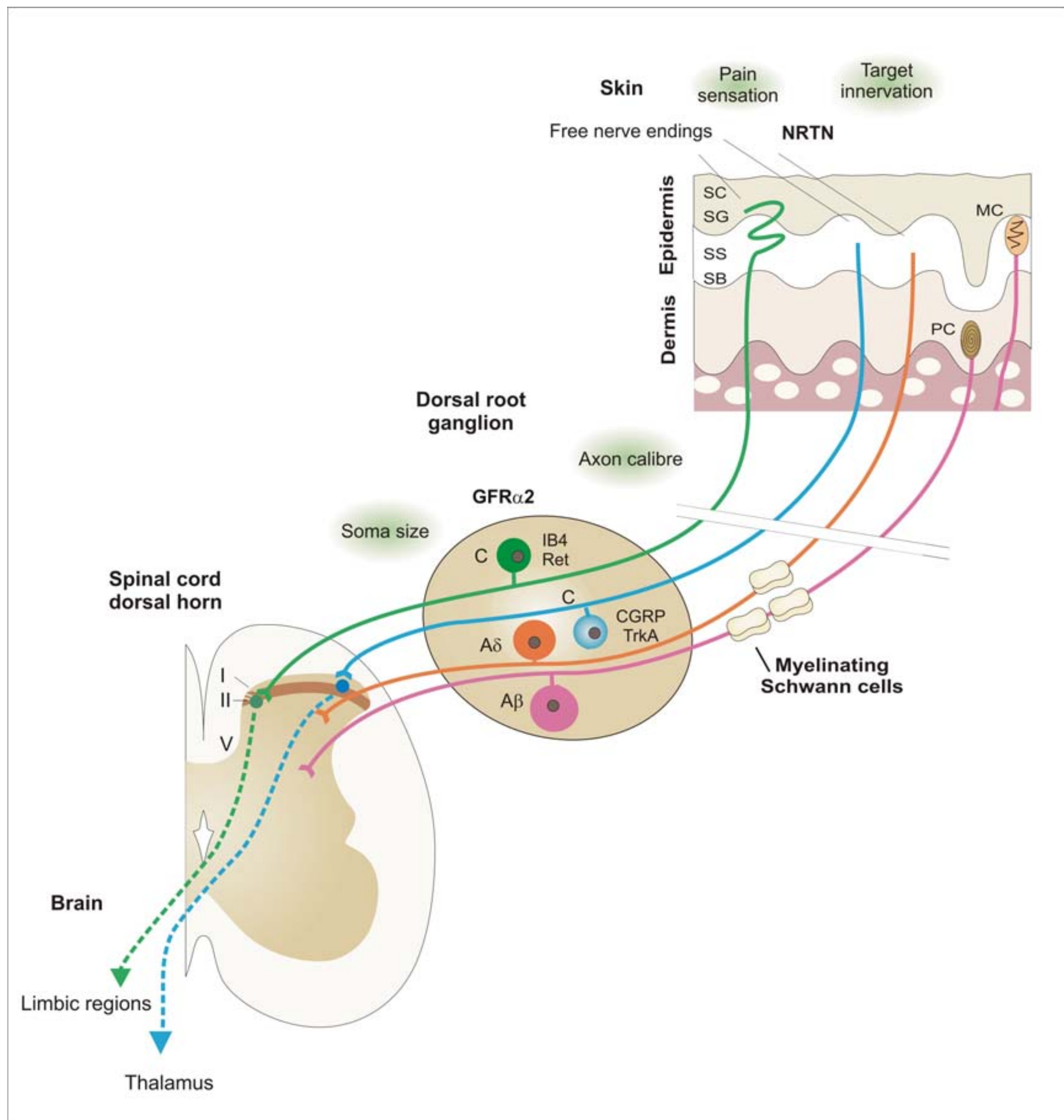

Figure 7. *In vivo* functions of GDNF family factors in somatosensory neurons. Cell bodies of sensory neurons are located in the dorsal root ganglia (DRG). Within the DRG, there are subclasses of sensory neurons, including proprioceptive and low-threshold mechanosensitive neurons, which have specialized nerve endings (e.g. Pacinian corpuscle; Meissner's corpuscle) and myelinated axons (Aβ). Pain and temperature-sensing neurons have lightly myelinated (Aδ) or unmyelinated C-fibres and terminate as free nerve endings. Unmyelinated nociceptors (C) can be subdivided into a peptidergic group, which expresses CGRP and TrkA, and non-peptidergic group, which binds IB_4 and expresses Ret and GFRα2. Peptidergic and non-peptidergic unmyelinated nociceptors terminate in different epidermal zones and different laminae of the spinal cord. Furthermore, they project to different brain areas. NRTN/GFRα2/Ret signalling is essential for the maintenance of soma size, axon calibre and terminal innervation of IB_4-binding unmyelinated nociceptors as well as for proper pain sensation. SB= stratum basalis, SC= stratum corneum, SG= stratum granulosum, SS= stratum spinosum, PC= Pacinian corpuscle, MC= Meissner's corpuscle. Adapted from Airaksinen and Saarma (2002), Patapoutian et al. (2003) and Zylka et al. (2005).

C. Phenotypes of GFRα2-deficient mice reflect the impaired target innervation

Functional deficits contributing to growth retardation in GFRα2-KO mice (I)

After pups are weaned and begin eating solid food, GFRα2-deficient mice exhibit growth retardation, which is most pronounced at the age of 4-6 weeks (Rossi et al., 1999). We analysed several factors that could contribute to the observed impaired growth. Secretion of saliva is mostly mediated by parasympathetic innervation of the salivary glands (Garrett, 1987). Among other functions saliva assists in eating and swallowing by lubricating the mouth and dissolving and diluting the food (Randall et al., 1997). GFRα2-KO mice almost entirely lack parasympathetic innervation in the sublingual gland and most of the innervation in the parotid gland (Rossi et al., 2000), suggesting decreased food-induced saliva secretion in these mice. Consistent with this, on a normal dry pellet diet, water intake per body weight was increased (~20%) in KO mice compared with their wild-type littermates (I; Fig. 6b). Furthermore, wet mash feeding could partially restore the growth of GFRα2-KO mice. The mice gained weight faster with the hydrated food but still significantly slower, suggesting that other defects besides salivary gland dysfunction contribute to poor growth (I).

Parasympathetic cholinergic innervation is thought to mediate the physiological food-induced secretion of pancreatic enzymes via the vagus nerve (Owyang, 1996; Rogers et al., 1996). The reduced pancreatic innervation in GFRα2-KO mice may result in an altered pancreatic secretion *in vivo*. For this a centrally acting vagal stimulant, 2-deoxyglucose (2-DG) (Havel and Taborsky, 1989; Li et al., 1998) was used. While 2-DG significantly increased amylase and protein secretion in wild-type, in GFRα2-KO mice, no significant increase in pancreatic secretion was elicited (I; Fig. 5). To investigate whether the growth retardation in GFRα2-deficient mice could be due to fat malabsorbtion, the mice were fed a high-fat chow and then the stool fat was measured. GFRα2-KO mice had a significantly elevated faecal fat content compared with wild-type mice (I). This malabsorption in GFRα2-KO mice is most likely due to pancreatic insufficiency.

GFRα2-deficient mice have previously been demonstrated to have impaired contractility of the small intestine *in vitro* (Rossi et al., 1999). Since excitatory innervation is crucial for normal gut motility (Sang and Young, 1998), intestinal motility *in vivo* was studied using the charcoal transit test (Bianchi et al., 1983). The intragastrically delivered test material travelled a significantly (~25%) shorter distance in the GFRα2-KO mice compared with the wild-type littermates. Apparently at least part of the weakened *in vivo* motility is due to the impaired excitatory (SP-positive) innervation of the longitudinal and circular muscles (I), although impaired neurotransmitter release (Heuckeroth et al., 1999) may also contribute to the dysmotility in GFRα2-KO mice. These and previous (Rossi et al., 1999) results suggest that the growth retardation in GFRα2-deficient mice is at least partly due to weakened secretion of pancreatic enzymes and saliva, which leads to poor digestion of food and malabsorption. The significance of impaired propulsion in the small intestine is less clear, because a slower motility could rather be expected to promote growth by giving more time for nutrient absorption. For example, $P2X_3$-deficient mice were shown to have impaired peristalsis in the small intestine, but no difference in body weight compared to wild-type mice (Bian et al., 2003).

Locomotor activity and body temperature were normal in GFRα2-deficient mice, but their basal metabolic rate was elevated (I). We estimated the body fat ratio by weighing the gonadal fat pads and found that the average weight was 40% lower in GFRα2-KO mice than in wild-type mice. Since fat is less metabolically active than muscle, a higher muscle-to-fat ratio can lead to the increased basal metabolic rate observed in GFRα2-KO mice. Furthermore, the KO mice consumed slightly more food per unit of body mass (~8%), which may also be attributable to the increased basal metabolic rate (I; Fig. 6). A post-weaning growth retardation phenotype, similar to the GFRα2-deficient mice, has been observed in mice lacking muscarinic acetylcholine receptor gene for the M3 subtype (Matsui et al., 2000; Yamada et al., 2001). These M3-deficient mice exhibited impaired *in vitro* contractility of the small intestine as well as low level of induced salivation (Matsui et al., 2000). However, the M3-deficient mice were also eating less than their wild type littermates and had reduced levels of serum insulin and leptin, a hormone involved in regulating appetite and metabolism (Yamada et al., 2001). Regulatory centres in the hypothalamus control an animal's energy homeostasis and food consumption. The hypothalamus is also known to express GDNF family receptors, including *Gfra2* (Golden et al., 1998). Reduced appetite cannot, however, be the reason for the poor growth, since GFRα2-KO mice ate more than their littermates; the mutant animals try to compensate for their malnutrition by increasing food and water intake. This also suggests that, despite reduced saliva secretion, the mice have no severe problems in swallowing. Finally, it remains unclear why NRTN-deficient mice (Heuckeroth et al., 1999) do not exhibit growth retardation on the same diet and breeding background. Possibily, other ligands are involved in GFRα2 signalling *in vivo* (Wanigasekara et al., 2004). It would be informative to study whether NRTN-deficient mice have impaired pancreatic innervation and function.

Reduced inflammatory pain response and enhanced thermal avoidance in GFRα2-deficient mice (III)

IB_4-binding neurons isolated from GFRα2-KO mouse DRG have defects in heat transduction *in vitro* (Stucky et al., 2002). However, in the hot plate test of thermal pain, no significant difference was observed in escape latency between GFRα2-KO and wild-type mice at plate temperatures of 52˚C or 49˚C (III; Fig. 5A and not shown). Similar results were obtained using mice in hybrid as well as congenic B6 and 129 backgrounds. In addition, in the tail immersion test, no difference was seen in withdrawal latency at a water bath temperature of 52˚C (III; Fig. 5B). However, at 49˚C, GFRα2-KO mice exhibited significantly shorter withdrawal latencies than wild-type mice. Moreover, the mutants exhibited much shorter latencies in the cold water (4˚C) tail withdrawal test (III; Fig. 5C, D). Thus, the physiological significance of the reduced heat transduction observed *in vitro* (Stucky et al., 2002) remains unclear. Alternatively, acutely isolated sensory neuron cultures may represent an injury situation (Hökfelt et al., 2006). Our results show that the thermoreceptor TRPV1 is not expressed with GFRα2 in mouse DRG, and the density of TRPV1-positive neurons is unchanged in GFRα2-KO mice (III; Fig. 1 and not shown), which is consistent with the report that IB_4-binding neurons do not express TRPV1 in mice (Zwick et al., 2002; Woodbury et al., 2004). Consequently, receptors other than TRPV1 may transduce noxious heat in GFRα2-positive IB_4-binding nociceptors. NRTN/GFRα2 signalling in TRPV1-mediated thermal hyperalgesia nevertheless

remains possible since peripheral inflammation and neurotrophic factors, including GDNF, can increase TRPV1 expression and function in IB_4-binding sensory neurons (Amaya et al., 2004; Breese et al., 2005).

The lack of most of the free nerve endings did not have a gross effect on the sensing of innocuous warmth, since both wild type and GFRα2-deficient mice behaved similarly in a two-temperature choice test (III; Fig. 5E). The remaining innervation is probably sufficient to allow normal response to non-noxious temperatures. Sometimes normal behaviour is exhibited despite dramatic anatomical changes, as demonstrated by a study in which NGF-deficient mice were rescued by transgenic expression of NGF in the skin. The response of these mice to noxious thermal stimuli recovered fully, although the IB_4-binding sensory neuron population was only modestly restored (Harrison et al., 2004). Furthermore, we found that both genotypes responded similarly to tactile stimulation in a von Frey test of mechanical sensitivity (III; Supplementary figure), consistent with a previous report using *in vitro* skin-nerve preparations (Stucky et al., 2002).

The formalin test is used to assess acute tissue injury-induced pain. Subcutaneous injection of formalin causes local tissue damage and activates both myelinated and unmyelinated nociceptors (Puig and Sorkin, 1995). The response to formalin shows a biphasic behavioural reaction. The early or acute phase is thought to reflect direct activation of nociceptors, whereas the late or tonic phase is thought to represent central sensitization and/or ongoing inflammation-induced afferent input. GFRα2-KO mice have attenuated response to inflammatory pain (III; Fig. 5F). During the first phase, GFRα2-KO mice spent an equal length of time licking and shaking the affected paw compared to wild type mice. However, in the second phase, the formalin-induced pain behaviour was significantly (~60%) reduced in GFRα2-KO mice compared with wild-type mice. The formalin-induced swelling did not differ between the genotypes, indicating a similar degree of inflammation (III). The intact first-phase response in GFRα2-KO mice suggests that functional nociceptor terminals mediating the acute response are present in the KO mouse skin. NGF and peptidergic nociceptors are known to mediate inflammatory pain (Snider and McMahon, 1998; Woolf and Costigan, 1999; Hunt and Mantyh, 2001), but recent studies suggest that also non-peptidergic nociceptors take part in the inflammatory processes (Breese et al., 2005). Inflammation induces C fibre hyperactivity and ongoing activity of dorsal horn neurons (Pitcher and Henry, 2002), both of which are thought to be critical for the tonic phase of formalin response (Tjolsen et al., 1992; Puig and Sorkin, 1995; Taylor et al., 1995). However, the behavioural pain response involves a network of neurons and other cells and is modulated at several levels. Whether the reduced formalin-induced inflammatory pain response involves peripheral and/or central mechanisms remains to be determined.

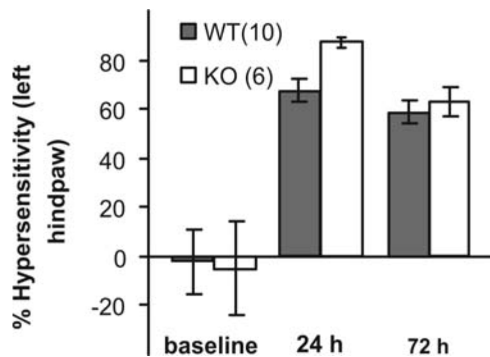

Figure 8. Response to chronic inflammatory pain. Thermal hyperalgesia to complete Freund's adjuvant is increased 24 h after the injection in GFRα2-KOmice. The results are shown as percentage hypersensitivity (injected paw/uninjected paw) before and 24 h (*p=0.003 using t-test) and 72 h after the adjuvant injection. The number of animals is indicates in parentheses.

Interestingly, formalin-induced inflammatory pain can be inhibited by specific $P2X_3$ blockers (Tsuda et al., 1999; McGaraughty et al., 2003) and is attenuated in $P2X_3$-deficient mice (Cockayne et al., 2000; Souslova et al., 2000). Moreover, the $P2X_3$-deficient mice exhibit a remarkably similar temperature sensation phenotype to the GFRα2-KO mice, including enhanced avoidance of noxious cold and hot temperatures in the tail withdrawal test (Shimizu et al., 2005). Contrary to reduced formalin-induced pain, the $P2X_3$-KO mice show markedly increased hyperalgesia in a model for chronic inflammatory pain (Souslova et al., 2000), and strikingly, our unpublished results suggest that GFRα2-deficient mice share this phenotype. Thermal hyperalgesia in response to complete Freund's adjuvant was transiently enhanced 24 h after the injection in GFRα2-deficient mice, but after 72 h, the genotypes responded similarly to the thermal stimulus (Fig. 8). Reason for this paradoxical potentiation of thermal hyperalgesia in both $P2X_3$-KO (Souslova et al., 2000) and GFRα2-KO mice following complete Freund's adjuvant is not known. Since GFRα2 and $P2X_3$ are co-localized extensively in mouse DRG, the reduced inflammatory pain response but enhanced hyperalgesia and thermal avoidance in GFRα2-KO mice could be caused by reduced activation of $P2X_3$. Another possible candidate mediator is bradykinin B1 receptor. Bradykinin is released during tissue damage and inflammation, and it causes an acute sensation of pain via B2 receptors expressed in nociceptors. Following injury or inflammation, the B1 receptor becomes upregulated, and this increased expression can be induced by NRTN (Vellani et al., 2004). NRTN-KO mice exhibit ocular surface inflammation, which resembles the human disease keratoconjunctivitis sicca (Song et al., 2003). This as well as the dry eyes phenotype observed in NRTN- and GFRα2-deficient mice are most likely due to impaired parasympathetic lacrimal gland innervation (Heuckeroth et al., 1999; Rossi et al., 1999). However, decreased tear production may also be caused by reduced afferent stimulation of tear production, and indeed NRTN-KO mice have been shown to have reduced sensitivity of polymodal sensory receptors in the cornea (Song et al., 2003). A summary of work III is illustrated in Fig. 7.

Table 3. Comparison of phenotypes of GFRα2- and NRTN-deficient mice.

Gene knockout	GFRα2-KO	NRTN-KO
Gross phenotype	Viable, fertile, pseudoptosis, growth retardation after weaning	Viable, fertile, pseudoptosis
Parasympathetic Sphenopalatine ganglion	Reduced soma size, lack of lacrimal gland innervation	Reduced soma size, lack of lacrimal gland innervation
Submandibular ganglion	42% reduced neuron number and size, reduced innervation of submandibular and sublingual gland	45% reduced neuron number, reduced innervation of submandibular and sublingual gland
Otic ganglion	40% reduced neuron number and size, reduced innervation of parotid gland	Reduced soma size
Ciliary ganglion	Neuron number not determined, intact innervation in pupillary constrictor muscle	40% reduced neuron number
Cardiac ganglia	Reduced innervation of heart, reduced volume of the cardiac ganglia	Not determined
Intrapancreatic ganglia	80% reduced neuron number and innervation of exocrine and endocrine pancreas	Not determined
Sacral ganglia	Reduced innervation of penis and the epithelium of reproductive organs. Smaller soma size of neurons innervating the bladder and vas deferens.	Reduced innervation of the epithelium of reproductive organs. Smaller soma size of neurons innervating the bladder and vas deferens.
Sympahetic Cholinergic neurons	Reduced soma size, reduced innervation of sweat glands and periosteum, normal number	Not determined
Noradrenergic neurons	Normal size and number and target innervation	Normal size and number and target innervation
Enteric	Reduced neuron number and reduced density of substanceP+ fibers in small intestine. Impaired gut motility *in vitro* and *in vivo*.	Reduced soma size, neuron number and density of substance P+ fibers in small intestine. Impaired gut motility *in vitro*. Reduced release of substance P from colon *in vitro*.
Sensory	Reduced soma size of IB_4-binding sensory neurons, smaller diameter of axons in saphenous nerve, reduced density of free nerve endings in the glabrous skin, loss of heat sensitivity in IB_4-binding neurons *in vitro*, reduced inflammatory pain response and enhanced thermal avoidance *in vivo*.	Loss of *Gfra2*-expressing sensory neurons in dorsal root – and trigeminal ganglia, reduced sensitivity of polymodal sensory receptors in the cornea.

D. *In vivo* function of GFRα4 (IV)

Generation of GFRα4-KO mice

To generate GFRα4-KO mice, a large part of the *Gfra4* gene was deleted using homologous recombination in embryonic stem cells and chimeric mice derived from these cells were bred to C57BL/6 and 129/Sv females to establish heterozygotes. GFRα4-deficient mice were born at the expected Mendelian frequency: of 288 offspring from heterozygous matings genotyped after weaning, 79 (27%) were wild type, 141 (49%) were *Gfra4+/-* and 68 (24%) were *Gfra4-/-*. The loss of *Gfra4* expression was confirmed by RT-PCR and with a polyclonal antibody for GFRα4 from several tissues (IV; Fig. 1, 3J). *In situ* hybridization with specific mouse RNA probes for *Gfra1*, *Gfra2*, *Gfra3* or *Ret* did not show signs of upregulation in a one-week-old GFRα4-KO thyroid (IV; Fig. 4). The GFRα4-KO mice were viable and fertile with normal growth and gross behaviour. Basic histological analysis of the central nervous system, pituitary gland and testis revealed no differences between wild-type and GFRα4-deficient mice. Moreover, no difference was observed between GFRα4-KO and wild type-mice in the distribution or number of TH- and PNMT-positive chromaffin cells in the adrenal medulla (IV, data not shown).

GFRα4 and Ret are co-localized exclusively in the juvenile mouse thyroid gland

Gfra4 mRNA is expressed in thyroid C-cells as well as in the parathyroid gland of juvenile mice (Lindahl et al., 2000). However, *in situ* hybridization using a probe recognizing the functional signal sequence of mouse *Gfra4* gene encoded by exon-1a gave a clear signal in the thyroid medulla, but no signal above background in the parathyroid gland (IV). Thus, in the thyro-parathyroid gland, only the C-cells express *Gfra4* transcripts with a functional signal sequence (IV; Fig. 2). There may be tissue-specific regulatory elements in the *Gfra4* promoter, or tissue-specific splicing of a short intron between exons 2 and 3 in *Gfra4* gene could restrict the production of a full-length protein (Lindahl et al., 2000). Addition of this intron results in *Gfra4* transcripts with premature stop codons (Lindahl et al., 2000), and translation of these transcripts produces a truncated, soluble protein that is secreted, or alternatively the transcripts undergo nonsense-mediated decay (Maquat, 2004). Expression of the GFRα4 protein was determined in different tissues known to express *Gfra4* mRNA (Lindahl et al., 2000) with an antibody for GFRα4 (IV; Fig. 3). GFRα4 immunoreactivity was seen in most but not all thyroid C-cells in newborn, juvenile and adult wild-type mouse, whereas no staining was observed in any other mouse tissue examined, including the adrenal gland, testis, pituitary gland, parathyroid gland and brain (IV; data not shown). Ret immunoreactivity, by contrast, decreased in the thyroid C-cells during development, being prominent at birth and virtually absent in adulthood (IV; Fig. 3). A similar decrease in the expression of *Ret* mRNA has been reported (Lindahl et al., 2000). Correctly spliced, full-length *Gfra4* mRNA has also been detected by RT-PCR in the pituitary intermediate lobe and the neonatal adrenal medulla (Lindahl et al., 2000), but no GFRα4 immunoreactivity was observed in these tissues (IV). One cannot, however, exclude the possibility that the GFRα4 protein is produced in these tissues at levels beyond detection by protein

immunohistochemistry. In conclusion, similarly to humans (Lindahl et al., 2001), GFRα4 expression appears to be restricted to thyroid C-cells in mice.

Normal development of thyroid C-cells in GFRα4-KO mice

In situ hybridization was carried out with specific probes for other GDNF family receptors (*Gfra1-3* and *Ret*) in a one-week-old wild-type mouse thyroid. *Ret* expression was seen in distinct clusters in the thyroid medulla, in a similar pattern to *Gfra4* mRNA expression reported previously (Belluardo et al., 1999; Lindahl et al., 2000). No expression of *Gfra1* or *Gfra3* mRNA was detected, but *Gfra2* mRNA was found in scattered cells across the thyroid gland. Double staining with CT and GFRα2 antibodies indicated that GFRα2 is not expressed in C-cells. These results show that juvenile mouse thyroid C-cells express *Ret* and *Gfra4* but no other GDNF family receptors (IV; Fig. 2, 4). As GFRα4 is the only GFRα receptor co-expressed with Ret in thyroid C-cells and since newborn Ret-deficient mice have ~37% less CT-positive cells than their wild-type littermates (Lindahl et al., 2000), we investigated whether GFRα4 is necessary for C-cell development. Immunostaining with an antibody against CT (a specific marker of C-cells) showed no differences in the morphology, distribution or number of thyroid C-cells between genotypes in newborn or adult mice (IV; Fig. 5). Thus, even if GFRα4 seems to be the only co-receptor expressed in thyroid C-cells with Ret in newborn and juvenile mice it is not needed for the early development of these cells. Although *Gfra1* is not expressed in thyroid C-cells postnatally (IV; Fig. 4), *in situ* hybridization of E12 embryos showed that the expression patterns of *Ret* and *Gfra1* mRNAs (in addition to *Gfra4*) (Lindahl et al., 2000) overlap in the ultimobranchial body, the structure from which the C-cells arise (IV; Fig. 6). Thus, the Ret-dependent subpopulation of C-cells may require signalling through GFRα1 during embryonic development. Confirmation of this hypothesis awaits studies using GFRα1 or GFRα1/GFRα4 double KO mice. The physiological significance of GFRα4 in the ultimobranchial body remains unclear, particularly as it is not known whether the *Gfra4* mRNA expressed in the mouse embryo at E12 codes for the functional form of GFRα4.

Reduced thyroid calcitonin levels and increased bone formation rate in newborn and juvenile GFRα4-KO mice

In contrast to normal C-cell development, the newborn and 3-week-old GFRα4-deficient mice had significantly reduced thyroid CT levels. Using an immunoradiometric assay, thyroid tissue CT levels were reduced by 60% in newborn and by 45% in juvenile GFRα4-KO mice (IV; Fig. 7). By contrast, thyroid CT levels in adult mice were similar between genotypes. These results were obtained from mice in both B6 and hybrid backgrounds. However, this phenotype appeared to be dependent on genetic background, since newborn GFRα4-KO mice in a hybrid 129/B6 background had an even more pronounced reduction in CT levels (80%), but newborn mice in a B6 background had almost similar CT levels between the genotypes. Likewise, a background effect was proposed for the incomplete penetrance of the ptosis phenotype in ARTN and GFRα3-KO mice (Honma et al., 2002). Furthermore, serum CT levels were reduced by ~40% in juvenile GFRα4-KO mice in

a hybrid 129/B6 background, but this did not reach statistical significance (data not shown). These results indicate an active role for GFRα4 in modulating CT production, and it is unlikely that the reduced CT levels are secondary due to developmental effects since the reduction was seen specifically in young, not adult, mice (IV; Fig. 7). Moreover, the lower thyroid CT levels in juvenile GFRα4-KO mice cannot be attributed to increased CT secretion since serum CT levels were also reduced (IV). Because *GFRA4* and *RET* mRNAs are co-expressed also in human thyroid C-cells (Lindahl et al., 2001), GFRA4/RET signalling may enhance CT production in humans. Although exogenous CT is known to inhibit bone resorption (Friedman and Raisz, 1965), CT, as well as CT receptor-deficient mice display an increased bone formation rate compared with their wild-type littermates (Hoff et al., 2002; Dacquin et al., 2004). Consistent with this, we observed a similar but milder increase (~20%) in bone formation in juvenile GFRα4-KO mice (IV; Fig. 8).

In contrast to CT levels, we found that the tissue adrenalin (WT = 14 ± 2 μg/ml, KO = 20 ± 1 μg/ml, $p = 0.02$, $n = 9$ for both genotypes) and noradrenalin (WT = 7 ± 1 μg/ml, KO = 11 ± 1 μg/ml, $p = 0.02$, $n = 9$ for both genotypes) levels measured by HPLC were slightly but significantly elevated in GFRα4-deficient adrenal glands (Hiltunen et al., 2001). However, the mechanism remains unclear, since we could not detect GFRα4 immunoreactivity in the mouse adrenal gland at any time point examined (IV, data not shown). But the increase in adrenalin and noradrenalin levels may be due to altered synthesis or release or chronic stress causing a compensatory upregulation of catecholamine syntesis. In contrast, Ret-deficient mice showed a ~30% reduced adrenaline, but not noradrenaline levels (Allmendinger et al., 2003).

GFRα4 is required for CT production in young mice only likely because expression levels of Ret mRNA (Lindahl et al., 2000) and protein (IV; Fig. 3) are higher in newborn and juvenile C-cells than in adult C-cells. In contrast, GFRα4 protein expression appears to persist in adult C-cells. Moreover, semiquantitative RT-PCR analysis amplifying the full-length *Gfra4* mRNA suggests that the relative expression of the functional GPI-anchored isoform (Yang et al., 2004) and the transmembrane isoform (Yang et al., 2005) of mouse GFRα4 is similar between newborn, juvenile and adult thyroid C-cells. The mechanism of GFRα4-mediated Ret signalling in regulating thyroid CT levels remains to be elucidated. One possibility suggested by our preliminary results is that it regulates CT mRNA levels: thyroid tissue CT mRNA expression appears to be reduced in juvenile GFRα4-KO mice compared with wild-type controls (IV; Suppl. Fig. 1). Signal transduction pathways and mechanisms that regulate CT production are not well known. Increased calcium levels stimulate CT secretion via G-protein-coupled calcium-sensing receptors (CaR) (Brown and MacLeod, 2001; Fudge and Kovacs, 2004) and also CT gene expression is stimulated by elevated calcium, presumably via calcium-dependent activation of transcription factor TTF-1 (Suzuki et al., 1998). CaR activation triggers the PI3-Kβ signalling pathway and the downstream effector PKCζ, which results in increased secretion of CT from cultured thyroid C-cells (Liu et al., 2003). Interestingly, tyrosine kinase receptors have been shown to stimulate G-protein-coupled receptors to activate the ERK/MAPK pathway (Brown and MacLeod, 2001). Therefore, one potential mechanism of how GFRα4 might regulate CT synthesis could be to modulate the CaR signal transduction by Ret. C-cells express the PI3-kinase isoform (Liu et al., 2003), which could be synergistically activated through tyrosine kinase and G-protein-coupled receptors. The function of PSPN in CT production requires clarification; however, our preliminary data indicate that CT levels are also decreased in juvenile

PSPN-KO mice (WT = 277 ± 136 ng/ml, KO = 137 ± 7 ng/ml, n = 7, $p < 0.0001$). Furthermore, it would be interesting to know whether enhanced Ret signalling in MTC caused by activating mutations in *RET* increases CT production in C-cells. Our results indicate that PSPN is able to activate phosphorylation of endogenous RET and downstream signalling in an established C-cell line with a MEN2A mutation in RET (IV; Suppl. Fig. 2).

The spliced transcript of *Pspn* is barely detectable in the mouse thyroid and brain, in contrast to several other tissues such as the adrenal gland and fat (IV; Suppl. Fig. 3). Unlike other GDNF family ligands, PSPN does not bind to heparan sulfate proteoglycans in the tissue matrix and therefore could circulate through body fluids (Bespalov and Saarma, unpublished data). Possibly, a secreted form of PSPN could be released from adrenal gland into the bloodstream and might act like a hormone, binding to GFRα4 on the C-cell surface. Lack of good antibodies has prevented from determining which cells produce PSPN in young mice and whether PSPN levels are sufficient to support this hypothesis. The findings that PSPN-deficient mice are hypersensitive to cerebral ischaemia and that this neuronal cell death can be prevented by PSPN (Tomac et al. 2002), suggest that functional GFRα4, the only known receptor for PSPN, is expressed in the brain. However, results presented in study IV strongly suggest that functional GFRα4 is not produced in the mouse brain (IV; Fig. 2E-H). On the other hand, *Gfra1-2* and *Ret* expression levels have been shown to increase in neurons after brain insults, such as kindling epilepsy and cerebral ischemia (Kokaia et al., 1999; Arvidsson et al., 2001). Thus, it is possible that GFRα4 is upregulated in the brain after lesions. Future studies will reveal whether GFRα4-KO mice have a similar ischaemia phenotype to PSPN-KO mice.

Chicken and mammalian GFRα4 differ in both their domain structure (Lindahl et al., 2000) and tissue expression patterns (Thompson et al., 1998; Homma et al., 2000, 2003), implying that their biological functions are also different. Chicken *Gfra4* is expressed in several neuronal populations in the developing CNS, including motoneurons (Homma et al., 2000, 2003). Mammalian motoneurons do not express *Gfra4* (Lindahl et al., 2000) and the only cells that express the functional form of GFRα4 in mouse seem to be the thyroid C-cells (IV). However, it is not known whether GFRα4 is expressed in ultimobranchial cells (source of CT in non-mammalian vertebrates) in chicken (Airaksinen et al., 2006). D1 domain that is missing from GFRα4 of placental mammals is present in GFRα4 of all other vertebrate classes (including marsupials) as well as in all other GFRα receptors (Airaksinen et al., 2006). Furthermore, although chicken GFRα4 is able to bind human PSPN (Enokido et al., 1998), there is no PSPN orthologue in the chicken genome (Airaksinen et al., 2006). Thus, compared with GFRα1 and GFRα2 (Airaksinen and Saarma, 2002), the biological function of GFRα4 may be less conserved in vertebrate evolution. GDNF has previously been shown to regulate transmitter release at neuromuscular junctions (Ribchester et al., 1998) and from dopamine neurons (Yang et al., 2001), and NRTN can induce the release of substance P from enteric neurons (Heuckeroth et al., 1999). The present study indicates that GFRα4-mediated Ret signalling may regulate the production of a transmitter *in vivo*, suggesting that signalling through other GFRα receptors may regulate transmitter synthesis in other cells.

CONCLUSIONS

1) GFRα2-mediated signalling was found to be essential for parasympathetic neuron survival and innervation in the exocrine pancreas and for a subpopulation of enteric neurons in the small intestine. The combination of impaired secretion of the salivary gland and the exocrine pancreas and possibly intestinal dysmotility contribute to growth retardation in GFRα2-KO mice (I).

2) The size and target innervation by cholinergic but not noradrenergic sympathetic neurons require GFRα2 (II).

3) GFRα2-mediated signalling is needed to maintain the size and terminal innervation of non-peptidergic cutaneous nociceptors *in vivo*. Deficient nociceptor development is associated with impaired inflammatory pain responses and enhanced thermal avoidance (III).

4) The functional form of GFRα4 seems to be restricted in thyroid C-cells of the mouse (IV).

5) Impaired thyroid calcitonin production is accompanied by increased bone formation in juvenile GFRα4-KO mice. The expression of this phenotype correlates with the co-expression of functional GFRα4 and Ret of C-cells in young mice (IV).

Taken together, the results presented here together with previous studies show that GFRα2/Ret signalling is crucial for the development of cholinergic autonomic neurons. These include most parasympathetic and cholinergic sympathetic neurons as well as a subpopulation of enteric neurons. Depending on the target tissue, GFRα2-mediated signalling is necessary for trophic support of the neurons and for target innervation or maintenance. Deficits in autonomic innervation lead to various functional impairments in GFRα2-KO mice. This study also established a novel role for GDNF family receptors, namely GFRα4, in regulating transmitter production. Thus, GFRα2/Ret signalling may have clinical potential in such conditions as intestinal dysmotility, exocrinopathies and neuropathic pain, and GFRα4/Ret signalling may be implicated in osteoporosis.

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REFERENCES

Ahren B (2000) Autonomic regulation of islet hormone secretion--implications for health and disease. Diabetologia 43: 393-410.

Airaksinen MS, Titievsky A, Saarma M (1999) GDNF family neurotrophic factor signaling: four masters, one servant? Mol Cell Neurosci 13: 313-325.

Airaksinen MS, Saarma M (2002) The GDNF family: signalling, biological functions and therapeutic value. Nature Rev Neurosci 3: 383-394.

Airaksinen MS, Holm L, Hätinen T (2006) Evolution of the GDNF family ligands and receptors. Brain Behav Evol, in press

Airavaara M, Mijatovic J, Vihavainen T, Piepponen TP, Saarma M, Ahtee L (2006) In heterozygous GDNF knockout mice the response of striatal dopaminergic system to acute morphine is altered. Synapse 59: 321-329.

Allmendinger A, Stoeckel E, Saarma M, Unsicker K, Huber K (2003) Development of adrenal chromaffin cells is largely normal in mice lacking the receptor tyrosine kinase c-Ret. Mech Dev 120: 299-304.

Amaya F, Shimosato G, Nagano M, Ueda M, Hashimoto S, Tanaka Y, Suzuki H, Tanaka M (2004) NGF and GDNF differentially regulate TRPV1 expression that contributes to development of inflammatory thermal hyperalgesia. Eur J Neurosci 20: 2303-2310.

Anders J, Kjær S, Ibáñez CF (2001) Molecular modeling of the extracellular domain of the RET receptor tyrosine kinase reveals multiple cadherin-like domains and a calcium-binding site. J Biol Chem 276: 35808-35817.

Anderson DJ (1993) Cell fate determination in the peripheral nervous system: the sympathoadrenal progenitor. J Neurobiol 24: 185-198.

Arenas E, Trupp M, Akerud P, Ibanez CF (1995) GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. Neuron 15: 1465-1473.

Arvidsson A, Kokaia Z, Airaksinen MS, Saarma M, Lindvall O (2001) Stroke induces widespread changes of gene expression for glial cell line-derived neurotrophic factor family receptors in the adult rat brain. Neuroscience 106:27-41.

Asmus SE, Parsons S, Landis SC (2000) Developmental changes in the transmitter properties of sympathetic neurons that innervate the periosteum. J Neurosci 20: 1495-1504.

Baker CVH, Bronner-Fraser (2000) Established neuronal identity in vertebrate neurogenic placodes. Development 127:3045-3056.

Baloh RH, Tansey MG, Golden JP, Creedon DJ, Heuckeroth RO, Keck CL, Zimonjic DB, Popescu NC, Johnson EM, Jr., Milbrandt J (1997) TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. Neuron 18: 793-802.

Baloh RH, Gorodinsky A, Golden JP, Tansey MG, Keck CL, Popescu NC, Johnson EM, Jr., Milbrandt J (1998a) GFRalpha3 is an orphan member of the GDNF/neurturin/persephin receptor family. Proc Natl Acad Sci U S A 95: 5801-5806.

Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, Simburger KS, Leitner ML, Araki T, Johnson EMJ, Milbrandt J (1998b) Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. Neuron 21: 1291-1302.

Baloh RH, Enomoto H, Johnson EMJ, Milbrandt J (2000) The GDNF family ligands and receptors - implications for neural development. Curr Opin Neurobiol 10: 103-110.

Barasch JM, Mackey H, Tamir H, Nunez EA, Gershon MD (1987a) Induction of a neural phenotype in a serotonergic endocrine cell derived from the neural crest. J Neurosci 7: 2874-2883.

Barasch JM, Tamir H, Nunez EA, Gershon MD (1987b) Serotonin-storing secretory granules from thyroid parafollicular cells. J Neurosci 7: 4017-4033.

Barnett MW, Fisher CE, Perona-Wright G, Davies JA (2002) Signalling by glial cell line-derived neurotrophic factor (GDNF) requires heparan sulphate glycosaminoglycan. J Cell Sci 115:4495-4503.

Baudet C, Mikaels A, Westphal H, Johansen J, Johansen TE, Ernfors P (2000) Positive and negative interactions of GDNF, NTN and ART in developing sensory neuron subpopulations, and their collaboration with neurotrophins. Development 127: 4335-4344.

Belluardo N, Mudo G, Caniglia G, Corsaro M, Cheng Q, Frasca F, Belfiore A, Condorelli DF (1999) Expression of neurotrophins, GDNF, and their receptors in rat thyroid tissue. Cell Tissue Res 295: 467-475.

Bennett DL, Michael GJ, Ramachandran N, Munson JB, Averill S, Yan Q, McMahon SB, Priestley JV (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. J Neurosci 18: 3059-3072.

Bennett DL, Boucher TJ, Armanini MP, Poulsen KT, Michael GJ, Priestley JV, Phillips HS, McMahon SB, Shelton DL (2000) The glial cell line-derived neurotrophic factor family receptor components are differentially regulated within sensory neurons after nerve injury. J Neurosci 20: 427-437.

Bian X, Ren J, DeVries M, Schnegelsberg B, Cockayne DA, Ford APDW, Galligan JJ (2003) Peristalsis is impaired in the small intestine of mice lacking the $P2X_3$ subunit. J Physiol 551:309-322.

Bianchi G, Ferretti P, Recchia M, Rocchetti M, Tavani A, Manara L (1983) Morphine tissue levels and reduction of gastrointestinal transit in rats. Correlation supports primary action site in the gut. Gastroenterology 85:852-858.

Björklund A, Kirik D, Rosenblad C, Georgievska B, Lundberg C, Mandel RJ (2000) Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. Brain Res 886: 82-98.

Bordeaux MC, Forcet C, Granger L, Corset V, Bidaud C, Billaud M, Bredesen DE, Edery P, Mehlen P (2000) The RET proto-oncogene induces apoptosis: a novel mechanism for Hirschsprung disease. EMBO J 19: 4056-4063.

Borrego S, Fernandez RM, Dziema H, Niess A, Lopez-Alonso M, Antinolo G, Eng C (2003) Investigation of germline GFRA4 mutations and evaluation of the involvement of GFRA1, GFRA2, GFRA3, and GFRA4 sequence variants in Hirschsprung disease. J Med Genet 40: e18.

Botchkareva NV, Botchkarev VA, Welker P, Airaksinen M, Roth W, Suvanto P, Muller-Rover S, Hadshiew IM, Peters C, Paus R (2000) New roles for glial cell line-derived neurotrophic factor and neurturin: involvement in hair cycle control. Am J Pathol 156: 1041-1053.

Boucher TJ, Okuse K, Bennett DL, Munson JB, Wood JN, McMahon SB (2000) Potent analgesic effects of GDNF in neuropathic pain states. Science 290: 124-127.

Bourque MJ, Trudeau LE (2000) GDNF enhances the synaptic efficacy of dopaminergic neurons in culture. Eur J Neurosci 12: 3172-3180.

Braz JM, Nassar MA, Wood JN, Basbaum AI (2005) Parallel "pain" pathways arise from subpopulations of primary afferent nociceptor. Neuron 47: 787-793.

Breese NM, George AC, Pauers LE, Stucky CL (2005) Peripheral inflammation selectively increases TRPV1 function in IB4-positive sensory neurons from adult mouse. Pain 115: 37-49.

Brodski C, Schnurch H, Dechant G (2000) Neurotrophin-3 promotes the cholinergic differentiation of sympathetic neurons. Proc Natl Acad Sci U S A 97: 9683-9688.

Brodski C, Schaubmar A, Dechant G (2002) Opposing functions of GDNF and NGF in the development of cholinergic and noradrenergic sympathetic neurons. Mol Cell Neurosci 19: 528-538.

Brown EM, MacLeod RJ (2001) Extracellular calcium sensing and extracellular calcium signaling. Physiol Rev 81: 239-297.

Brunicardi FC, Shavelle DM, Andersen DK (1995) Neural regulation of the endocrine pancreas. Int J Pancreatol 18: 177-195.

Buj-Bello A, Buchman VL, Horton A, Rosenthal A, Davies AM (1995) GDNF is an age-specific survival factor for sensory and autonomic neurons. Neuron 15: 821-828.

Buj-Bello A, Adu J, Pinon LG, Horton A, Thompson J, Rosenthal A, Chinchetru M, Buchman VL, Davies AM (1997) Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. Nature 387: 721-724.

Bullock SL, Fletcher JM, Beddington RS, Wilson VA (1998) renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. Genes dev 12:1894-1906.

Burau K, Stenull I, Huber K, Misawa H, Berse B, Unsicker K, Ernsberger U (2004) c-ret regulates cholinergic properties in mouse sympathetic neurons: evidence from mutant mice. Eur J Neurosci 20: 353-362.

Burek MJ, Oppenheim RW (1996) Programmed cell death in the developing nervous system. Brain Pathol 6: 427-446.

Burton MD, Kawashima A, Brayer JA, Kazemi H, Shannon DC, Schuchardt A, Costantini F, Pachnis V, Kinane TB (1997) RET proto-oncogene is important for the development of respiratory CO2 sensitivity. J Auton Nerv Syst 63: 137-143.

Cacalano G, Farinas I, Wang LC, Hagler K, Forgie A, Moore M, Armanini M, Phillips H, Ryan AM, Reichardt LF, Hynes M, Davies A, Rosenthal A (1998) GFRalpha1 is an essential receptor component for GDNF in the developing nervous system and kidney. Neuron 21: 53-62.

Califano D, Rizzo C, D'Alessio A, Colucci-D'Amato GL, Cali G, Bartoli PC, Santelli G, Vecchio G, de F, V (2000) Signaling through Ras is essential for ret oncogene-induced cell differentiation in PC12 cells. J Biol Chem 275: 19297-19305.

Camassei FD, Boldrini R, Jenkner A, Inserra A, Donfrancesco A, Rava L, Dominici C (2003) Expression of glial cell line-derived neurotrophic factor and neurturin in mature kidney, nephrogenic rests, and nephroblastoma: possible role as differentiating factors. Pediatr Dev Pathol 6(6):511-519.

Carrasquillo MM, McCallion AS, Puffenberger EG, Kashuk CS, Nouri N, Chakravarti A (2002) Genome-wide association study and mouse model identify interaction between RET and EDNRB pathways in Hirschsprung disease. Nat Genet 32: 237-244.

Caterina MJ, Rosen TA, Tominaga M, Brake AJ, Julius D (1999) A capsaicin-receptor homologue with a high threshold for noxious heat. Nature 398: 436-441.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288: 306-313

Chalazonitis A, Rothman TP, Chen J, Gershon MD (1998) Age-dependent differences in the effects of GDNF and NT-3 on the development of neurons and glia from neural crest-derived precursors immunoselected from the fetal rat gut: expression of GFRalpha-1 in vitro and in vivo. Dev Biol 204: 385-406.

Cherruau M, Morvan FO, Schirar A, Saffar JL (2003) Chemical sympathectomy-induced changes in TH-, VIP-, and CGRP-immunoreactive fibers in the rat mandible periosteum: influence on bone resorption. J Cell Physiol 194: 341-348.

Chien HF, Tseng TJ, Lin WM, Yang CC, Chang YC, Chen RC, Hsieh ST (2001) Quantitative pathology of cutaneous nerve terminal degeneration in the human skin. Acta Neuropathol (Berl) 102: 455-461.

Christianson JA, Riekhof JT, Wright DE (2003) Restorative effects of neurotrophin treatment on diabetes-induced cutaneous axon loss in mice. Exp Neurol 179: 188-199.

Civitelli R, Gonnelli S, Zacchei F, Bigazzi S, Vattimo A, Avioli LV, Gennari C (1988) Bone turnover in postmenopausal osteoporosis. Effect of calcitonin treatment. J Clin Invest 82: 1268-1274.

Clark MS, Lanigan TM, Page NM, Russo AF (1995) Induction of a serotonergic and neuronal phenotype in thyroid C-cells. J Neurosci 15: 6167-6178.

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB, Ford AP (2000) Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. Nature 407: 1011-1015.

Cowen T, Thrasivoulou C, Shaw SA, Abdel-Rahman TA (1996) Transplanted sweat glands from mature and aged donors determine cholinergic phenotype and altered density of host sympathetic nerves. J Auton Nerv Syst 60: 215-224.

Cremer H, Chazal G, Lledo PM, Rougon G, Montaron MF, Mayo W, Le Moal M, Abrous DN (2000) PSA-NCAM: an important regulator of hippocampal plasticity. Int J Dev Neurosci 18: 213-220.

Dacquin R, Davey RA, Laplace C, Levasseur R, Morris HA, Goldring SR, Gebre-Medhin S, Galson DL, Zajac JD, Karsenty G (2004) Amylin inhibits bone resorption while the calcitonin receptor controls bone formation in vivo. J Cell Biol 164: 509-514.

Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. Nature 405: 183-187.

de Graaff E, Srinivas S, Kilkenny C, D'Agati V, Mankoo BS, Costantini F, Pachnis V (2001) Differential activities of the RET tyrosine kinase receptor isoforms during mammalian embryogenesis. Genes Dev 15: 2433-2444.

De Vita G, Melillo RM, Carlomagno F, Visconti R, Castellone MD, Bellacosa A, Billaud M, Fusco A, Tsichlis PN, Santoro M (2000) Tyrosine 1062 of RET-MEN2A mediates activation of Akt (protein kinase B) and mitogen-activated protein kinase pathways leading to PC12 cell survival. Cancer Res 60: 3727-3731.

Dodd J, Role LW (1991) The autonomic nervous system. In principles of neural science. Kandel ER, Schwartz JH and Jessell TM (eds.), 3rd edition, Appleton & Lange, pp. 761-775

Duong CV, Geissen M, Rohrer H (2002) The developmental expression of vasoactive intestinal peptide (VIP) in cholinergic sympathetic neurons depends on cytokines signaling through LIFRbeta-containing receptors. Development 129: 1387-1396.

Durbec PL, Larsson-Blomberg LB, Schuchardt A, Costantini F, Pachnis V (1996) Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. Development 122: 349-358.

Easton RM, Deckwerth TL, Parsadanian AS, Johnson EMJ (1997) Analysis of the mechanism of loss of trophic factor dependence associated with neuronal maturation: a phenotype indistinguishable from Bax deletion. J Neurosci 17: 9656-9666.

Eigenbrot C, Gerber N (1997) X-ray structure of glial cell-derived neurotrophic factor at 1.9 A resolution and implications for receptor binding. Nat Struct Biol 4: 435-438.

Eketjäll S, Fainzilber M, Murray-Rust J, Ibáñez CF (1999) Distinct structural elements in GDNF mediate binding to GFRα1 and activation of the GFRα1-c-Ret receptor complex. EMBO J 18: 5901-5910.

Eketjall S, Ibanez CF (2002) Functional characterization of mutations in the GDNF gene of patients with Hirschsprung disease. Hum Mol Genet 11: 325-329.

ElShamy WM, Linnarsson S, Lee KF, Jaenisch R, Ernfors P (1996) Prenatal and postnatal requirements of NT-3 for sympathetic neuroblast survival and innervation of specific targets. Development 122: 491-500.

Encinas M, Crowder RJ, Milbrandt J, Johnson EMJ (2004) Tyrosine 981, a novel Ret autophosphorylation site, binds c-Src to mediate neuronal survival. J Biol Chem 279(18):18262-18269.

Enokido Y, de Sauvage F, Hongo JA, Ninkina N, Rosenthal A, Buchman VL, Davies AM (1998) GFRα-4 and the tyrosine kinase Ret form a functional receptor complex for persephin. Curr Biol 8: 1019-1022.

Enomoto H, Araki T, Jackman A, Heuckeroth RO, Snider WD, Johnson EMJ, Milbrandt J (1998) GFRa1-deficient mice have deficits in the enteric nervous system and kidneys. Neuron 21: 317-324.

Enomoto H, Heuckeroth RO, Golden JP, Johnson EM, Milbrandt J (2000) Development of cranial parasympathetic ganglia requires sequential actions of GDNF and neurturin. Development 127: 4877-4889.

Enomoto H, Crawford PA, Gorodinsky A, Heuckeroth RO, Johnson EMJ, Milbrandt J (2001) RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development 128: 3963-3974.

Enomoto H, Hughes I, Golden J, Baloh RH, Yonemura S, Heuckeroth RO, Johnson EMJ, Milbrandt J (2004) GFRα1 expression in cells lacking RET is dispensable for organogenesis and nerve regeneration. Neuron 44:623-636.

Enomoto H (2005) Regulation of neural development by glial cell line-derived neurotrophic factor family ligands. Anat Sci Int 80:42-52.

Erickson JT, Brosenitsch TA, Katz DM (2001) Brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor are required simultaneously for survival of dopaminergic primary sensory neurons in vivo. J Neurosci 21: 581-589.

Ernfors P, Lee KF, Kucera J, Jaenisch R (1994) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. Cell 20;77: 503-512.

Ernsberger U, Reissmann E, Mason I, Rohrer H (2000) The expression of dopamine beta-hydroxylase, tyrosine hydroxylase, and Phox2 transcription factors in sympathetic neurons: evidence for common regulation during noradrenergic induction and diverging regulation later in development. Mech Dev 92: 169-177.

Ernsberger U, Rohrer H (1999) Development of the cholinergic neurotransmitter phenotype in postganglionic sympathetic neurons. Cell Tissue Res 297: 339-361.

Farinas I, Cano-Jaimez M, Bellmunt E, Soriano M (2002) Regulation of neurogenesis by neurotrophins in developing spinal sensory ganglia. Brain Res Bull 57: 809-816.

Fields HL, Rowbotham M, Baron R (1998) Postherpetic neuralgia: irritable nociceptors and deafferentation. Neurobiol Dis 5: 209-227.

Fisher CE, Michael L, Barnett MW, Davies JA (2001) Erk MAP kinase regulates branching morphogenesis in the developing mouse kidney. Development 128: 4329-4338.

Fitze G, Paditz E, Schlafke M, Kuhlisch E, Roesner D, Schackert HK (2003) Association of germline mutations and polymorphisms of the RET proto-oncogene with idiopathic congenital central hypoventilation syndrome in 33 patients. J Med Genet 40: E10.

Fontaine J (1979) Multistep migration of calcitonin cell precursors during ontogeny of the mouse pharynx. Gen Comp Endocrinol 37: 81-92.

Forander P, Broberger C, Stromberg I (2001) Glial-cell-line-derived neurotrophic factor induces nerve fibre formation in primary cultures of adrenal chromaffin cells. Cell Tissue Res 305: 43-51.

Forgie A, Doxakis E, Buj-Bello A, Wyatt S, Davies AM (1999) Differences and developmental changes in the responsiveness of PNS neurons to GDNF and neurturin. Mol Cell Neurosci 13: 430-440.

Francis NJ, Asmus SE, Landis SC (1997) CNTF and LIF are not required for the target-directed acquisition of cholinergic and peptidergic properties by sympathetic neurons in vivo. Dev Biol 182: 76-87.

Francis NJ, Landis SC (1999) Cellular and molecular determinants of sympathetic neuron development. Annu Rev Neurosci 22: 541-566.

Francis N, Farinas I, Brennan C, Rivas-Plata K, Backus C, Reichardt L, Landis S (1999) NT-3, like NGF, is required for survival of sympathetic neurons, but not their precursors. Dev Biol 210: 411-427.

Friedman J, Raisz LG (1965) Thyrocalcitonin: inhibitor of bone resorption in tissue culture. Science 150: 1465-1467.

Fudge NJ, Kovacs CS (2004) Physiological studies in heterozygous calcium sensing receptor (CaSR) gene-ablated mice confirm that the CaSR regulates calcitonin release in vivo. BMC Physiol 20;4: 5.

Fukuda T, Kiuchi K, Takahashi M (2002) Novel mechanism of regulation of Rac activity and lamellipodia formation by RET tyrosine kinase. J Biol Chem 277(21):19114-19121.

Fundin BT, Mikaels A, Westphal H, Ernfors P (1999) A rapid and dynamic regulation of GDNF-family ligands and receptors correlate with the developmental dependency of cutaneous sensory innervation. Development 126: 2597-2610.

Garces A, Livet J, Grillet N, Henderson CE, deLapeyriere O (2001) Responsiveness to neurturin of subpopulations of embryonic rat spinal motoneuron does not correlate with expression of GFR alpha 1 or GFR alpha 2. Dev Dyn 220: 189-197.

Gardell LR, Wang R, Ehrenfels C, Ossipov MH, Rossomando AJ, Miller S, Buckley C, Cai AK, Tse A, Foley SF, Gong B, Walus L, Carmillo P, Worley D, Huang C, Engber T, Pepinsky B, Cate RL, Vanderah TW, Lai J, Sah DW, Porreca F (2003) Multiple actions of systemic artemin in experimental neuropathy. Nat Med 9(11):1383-1389.

Garrett JR (1987) The proper role of nerves in salivary secretion: a review. J Dent Res 66: 387-397.

Gerlai R, McNamara A, Choi-Lundberg DL, Armanini M, Ross J, Powell-Braxton L, Phillips HS (2001) Impaired water maze learning performance without altered dopaminergic function in mice heterozygous for the GDNF mutation. Eur J Neurosci 14: 1153-1163.

Gianino S, Grider JR, Cresswell J, Enomoto H, Heuckeroth RO (2003) GDNF availability determines enteric neuron number by controlling precursor proliferation. Development 130: 2187-2198.

Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN, Heywood P (2003) Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat Med 9: 589-595.

Glebova NO, Ginty DD (2004) Heterogeneous requirement of NGF for sympathetic target innervation in vivo. J Neurosci 24: 743-751.

Glebova NO, Ginty DD (2005) Growth and survival signals controlling sympathetic nervous system development. Annu Rev Neurosci 28:191-222.

Golden JP, Baloh RH, Kotzbauer PT, Lampe PA, Osborne PA, Milbrandt J, Johnson EM, Jr. (1998) Expression of neurturin, GDNF, and their receptors in the adult mouse CNS. J Comp Neurol 398: 139-150.

Golden JP, DeMaro JA, Osborne PA, Milbrandt J, Johnson EM, Jr. (1999) Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. Exp Neurol 158: 504-528.

Golden JP, Milbrandt J, Johnson EM (2003) Neurturin and persephin promote the survival of embryonic basal forebrain cholinergic neurons in vitro. Exp Neurol 184: 447-455.

Goridis C, Rohrer H (2002) Specification of catecholaminergic and serotonergic neurons. Nat Rev Neurosci 3:531-541.

Granholm AC, Reyland M, Albeck D, Sanders L, Gerhardt G, Hoernig G, Shen L, Westphal H, Hoffer B (2000) Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. J Neurosci 20: 3182-3190.

Grondin R, Cass WA, Zhang Z, Stanford JA, Gash DM, Gerhardt GA (2003) Glial cell line-derived neurotrophic factor increases stimulus-evoked dopamine release and motor speed in aged rhesus monkeys. J Neurosci 23: 1974-1980.

Grundy D, Schemann M (2005) Enteric nervous system. Curr Opin Gastroenterol 21: 176-182.

Guidry G, Landis SC (1995) Sympathetic axons pathfind successfully in the absence of target. J Neurosci 15: 7565-7574.

Guidry G, Landis SC (1998) Target-dependent development of the vesicular acetylcholine transporter in rodent sweat gland innervation. Dev Biol 199: 175-184.

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL (1993) Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75: 463-476.

Habecker BA, Landis SC (1994) Noradrenergic regulation of cholinergic differentiation. Science 264: 1602-1604.

Habecker BA, Symes AJ, Stahl N, Francis NJ, Economides A, Fink JS, Yancopoulos GD, Landis SC (1997) A sweat gland-derived differentiation activity acts through known cytokine signaling pathways. J Biol Chem 272: 30421-30428.

Hansford JR, Mulligan LM (2000) Multiple endocrine neoplasia type 2 and RET: from neoplasia to neurogenesis. J Med Genet 37: 817-827.

Hargreaves KM, Dubner R, Brown F, Flores C, Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32:77-88.

Harrison SM, Davis BM, Nishimura M, Albers KM, Jones ME, Phillips HS (2004) Rescue of NGF-deficient mice I: transgenic expression of NGF in skin rescues mice lacking endogenous NGF. Brain Res Mol Brain Res 122: 116-125.

Hase A, Suzuki H, Arahata K, Akazawa C (1999) Expression of human GFRα1 (GDNF receptor) at the neuromuscular junction and myelinated nerves. Neurosci Lett 269:55-57.

Hase A, Saito F, Yamada H, Arai K, Shimizu T, Matsumura K (2005) Characterization of glial cell line-derived neurotrophic factor family receptor α-1 in peripheral nerve Schwann cells. J Neurochem 95:537-543.

Havel PJ, Taborsky GJJ (1989) The contribution of the autonomic nervous system to changes of glucagon and insulin secretion during hypoglycemic stress. Endocr Rev 10: 332-350.

Hayashi H, Ichihara M, Iwashita T, Murakami H, Shimono Y, Kawai K, Kurokawa K, Murakumo Y, Imai T, Funahashi H, Nakao A, Takahashi M (2000) Characterization of intracellular signals via tyrosine 1062 in RET activated by glial cell line-derived neurotrophic factor [In Process Citation]. Oncogene 19: 4469-4475.

Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C, Armanini M, Simmons L, Moffet B, Vandlen RA, Simmons L (1994) GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 266: 1062-1064.

Herrmann DN, Griffin JW, Hauer P, Cornblath DR, McArthur JC (1999) Epidermal nerve fiber density and sural nerve morphometry in peripheral neuropathies. Neurology 53: 1634-1640.

Heuckeroth RO, Lampe PA, Johnson EM, Milbrandt J (1998) Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. Dev Biol 200: 116-129.

Heuckeroth RO, Enomoto H, Grider JR, Golden JP, Hanke JA, Jackman A, Molliver DC, Bardgett ME, Snider WD, Johnson EMJ, Milbrandt J (1999) Gene targeting reveals a critical role for neurturin in the development and

maintenance of enteric, sensory, and parasympathetic neurons. Neuron 22: 253-263.

Hiltunen JO, Laurikainen A, Airaksinen MS, Saarma M (2000) GDNF family receptors in the embryonic and postnatal rat heart and reduced cholinergic innervation in mice hearts lacking ret or GFRα2. Dev Dyn 219: 28-39.

Hiltunen P, Lindahl M, Rossi J, Piepponen P, Timmusk T, Saarma M, Airaksinen MS (2001) Initial characterization of GDNF-family receptor GFRα4-deficient mice. Soc Neurosci Abstr 27: Progr no 364.31.

Hoff AO, Catala-Lehnen P, Thomas PM, Priemel M, Rueger JM, Nasonkin I, Bradley A, Hughes MR, Ordonez N, Cote GJ, Amling M, Gagel RF (2002) Increased bone mass is an unexpected phenotype associated with deletion of the calcitonin gene. J Clin Invest 110: 1849-1857.

Hohmann EL, Elde RP, Rysavy JA, Einzig S, Gebhard RL (1986) Innervation of periosteum and bone by sympathetic vasoactive intestinal peptide-containing nerve fibers. Science 232: 868-871.

Holland NR, Stocks A, Hauer P, Cornblath DR, Griffin JW, McArthur JC (1997) Intraepidermal nerve fiber density in patients with painful sensory neuropathy. Neurology 48: 708-711.

Homma S, Oppenheim RW, Yaginuma H, Kimura S (2000) Expression pattern of GDNF, c-ret, and GFRalphas suggests novel roles for GDNF ligands during early organogenesis in the chick embryo. Dev Biol 217: 121-137.

Homma S, Yaginuma H, Vinsant S, Seino M, Kawata M, Gould T, Shimada T, Kobayashi N, Oppenheim RW (2003) Differential expression of the GDNF family receptors RET and GFRalpha1, 2, and 4 in subsets of motoneurons: A relationship between motoneuron birthdate and receptor expression. J Comp Neurol 456: 245-259.

Honma Y, Araki T, Gianino S, Bruce A, Heuckeroth R, Johnson E, Milbrandt J (2002) Artemin is a vascular-derived neurotropic factor for developing sympathetic neurons. Neuron 35: 267-282.

Horger BA, Nishimura MC, Armanini MP, Wang LC, Poulsen KT, Rosenblad C, Kirik D, Moffat B, Simmons L, Johnson E, Jr., Milbrandt J, Rosenthal A, Bjorklund A, Vandlen RA, Hynes MA, Phillips HS (1998) Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurons. J Neurosci 18: 4929-4937.

Howard MJ (2005) Mechanisms and perspectives on differentiation of autonomic neurons. Dev Biol 277:271-286.

Huang EJ, Reichardt LF (2001) Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci 24: 677-736.

Huber K, Bruhl B, Guillemot F, Olson EN, Ernsberger U, Unsicker K (2002) Development of chromaffin cells depends on MASH1 function. Development 129: 4729-4738.

Huber K, Karch N, Ernsberger U, Goridis C, Unsicker K (2005) The role of Phox2B in chromaffin cell development. Dev Biol 279: 501-508.

Hucho TB, Dina OA, Levine JD (2005) Epac mediates a cAMP-to-PKC signaling in inflammatory pain: an isolectin B4(+) neuron-specific mechanism. J Neurosci 25: 6119-6126.

Hunt SP, Mantyh PW (2001) The molecular dynamics of pain control. Nat Rev Neurosci 2: 83-91.

Hökfelt TGM, Zhang X, Xu X-J, Wiesenfeld-Hallin Z (200) Central consequences of peripheral nerve damage. Wall and Melzack's textbook of pain. 5th edition, McMahon SB and Koltzenburg M (eds.), Elsevier, pp. 947-960.

Ichihara M, Murakumo Y, Takahashi M (2004) RET and neuroendocrine tumors. Cancer Lett 20;204: 197-211.

Iwamoto T, Taniguchi M, Asai N, Ohkusu K, Nakashima I, Takahashi M (1993) cDNA cloning of mouse ret proto-oncogene and its sequence similarity to the cadherin superfamily. Oncogene 8: 1087-1091.

Jing S, Wen D, Yu Y, Holst PL, Luo Y, Fang M, Tamir R, Antonio L, Hu Z, Cupples R, Louis JC, Hu S, Altrock BW, Fox GM (1996) GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF. Cell 85: 1113-1124.

Kato M, Takeda K, Kawamoto Y, Tsuzuki T, Dai Y, Nakayama S, Toriyama K, Tamada Y, Takahashi M, Nakashima I (2001) RET tyrosine kinase enhances hair growth in association with promotion of melanogenesis. Oncogene 20: 7536-7541.

Kawai K, Iwashita T, Murakami H, Hiraiwa N, Yoshiki A, Kusakabe M, Ono K, Iida K, Nakayama A, Takahashi M (2000) Tissue-specific carcinogenesis in transgenic mice expressing the RET proto-oncogene with a multiple endocrine neoplasia type 2A mutation. Cancer Res 60: 5254-5260.

Kawamoto Y, Nakamura S, Matsuo A, Akiguchi I, Shibasaki H (2000) Immunohistochemical localization of glial cell line-derived neurotrophic factor in the human central nervous system. Neuroscience 100(4):701-712.

Kim SK, MacDonald RJ (2002) Signaling and transcriptional control of pancreatic organogenesis. Curr Opin Genet Dev 12: 540-547.

Kirchgessner AL, Gershon MD (1990) Innervation of the pancreas by neurons in the gut. J Neurosci 10: 1626-1642.

Kirchgessner AL, Adlersberg MA, Gershon MD (1992) Colonization of the developing pancreas by neural precursors from the bowel. Dev Dyn 194: 142-154.

Klein RD, Sherman D, Ho WH, Stone D, Bennett GL, Moffat B, Vandlen R, Simmons L, Gu Q, Hongo JA, Devaux B, Poulsen K, Armanini M, Nozaki C, Asai N, Goddard A, Phillips H, Henderson CE, Takahashi M, Rosenthal A (1997) A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature 387: 717-721.

Kokaia Z, Airaksinen MS, Nanobashvili A, Larsson E, Kujamäki E, Lindvall O, Saarma M (1999) GDNF family ligands and receptors are differentially regulated after brain insults in the rat. Eur J Neurosci 11:1202-1216.

Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, McBride J, Chen EY, Palfi S, Roitberg BZ, Brown WD, Holden JE, Pyzalski R, Taylor MD, Carvey P, Ling Z, Trono D, Hantraye P, Deglon N, Aebischer P (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science 290: 767-773.

Korsching S (1993) The neurotrophic factor concept: a reexamination. J Neurosci 13: 2739-2748.

Kotzbauer PT, Lampe PA, Heuckeroth RO, Golden JP, Creedon DJ, Johnson EM, Jr., Milbrandt J (1996) Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature 384: 467-470.

Krieglstein K, Deimling F, Suter-Crazzolara C, Unsicker K (1996) Expression and localization of GDNF in developing and adult adrenal chromaffin cells. Cell Tissue Res 286: 263-268.

Krieglstein K, Henheik P, Farkas L, Jaszai J, Galter D, Krohn K, Unsicker K (1998) Glial cell line-derived neurotrophic factor requires transforming growth factor-beta for exerting its full neurotrophic potential on peripheral and CNS neurons. J Neurosci 18: 9822-9834.

Kuma K, Iwabe N, Miyata T (1993) Motifs of cadherin- and fibronectin type III-related sequences and evolution of the receptor-type-protein tyrosine kinases: sequence similarity between proto-oncogene ret and cadherin family. Mol Biol Evol 10: 539-551.

Kurokawa K, Kawai K, Hashimoto M, Ito Y, Takahashi M (2003) Cell signalling and gene expression mediated by RET tyrosine kinase. J Intern Med 253: 627-633.

Kuruvilla R, Zweifel LS, Glebova NO, Lonze BE, Valdez G, Ye H, Ginty DD (2004) A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell 118: 243-255.

Landis SC, Keefe D (1983) Evidence for neurotransmitter plasticity in vivo: developmental changes in properties of cholinergic sympathetic neurons. Dev Biol 98: 349-372.

Lang AE, Gill S, Patel NK, Lozano A, Nutt JG, Penn R, Brooks DJ, Hotton G, Moro E, Heywood P, Brodsky MA, Burchiel K, Kelly P, Dalvi A, Scott B, Stacy M, Turner D, Wooten VG, Elias WJ, Laws ER, Dhawan V, Stoessl AJ, Matcham J, Coffey RJ, Traub M (2006) Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. Ann Neurol 59(3):459-466.

Lanigan TM, DeRaad SK, Russo AF (1998) Requirement of the MASH-1 transcription factor for neuroendocrine differentiation of thyroid C cells. J Neurobiol 34: 126-134.

Laurikainen A, Hiltunen JO, Thomas-Crusells J, Vanhatalo S, Arumae U, Airaksinen MS, Klinge E, Saarma M (2000) Neurturin is a neurotrophic factor for penile parasympathetic neurons in adult rat. J Neurobiol 43: 198-205.

Lecci A, Santicioli P, Maggi CA (2002) Pharmacology of transmission to gastrointestinal muscle. Curr Opin Pharmacol 2: 630-641.

Ledda F, Paratcha G, Ibanez CF (2002) Target-derived GFRalpha1 as an attractive guidance signal for developing sensory and sympathetic axons via activation of Cdk5. Neuron 36: 387-401.

Lee H, Iida T, Mizuno A, Suzuki M, Caterina MJ (2005) Altered thermal selection behavior in mice lacking transient receptor potential vanilloid 4. J Neurosci 25: 1304-1310.

Lee H-Y, Kleber M, Hari L, Brault V, Suter U, Taketo MM, Kemler R, Sommer L (2004) Instructive role of Wnt/β-catenin in sensory fate specification in neural crest stem cells. Science 303:1020-1023.

Leppänen VM, Bespalov MM, Runeberg-Roos P, Puurand U, Merits A, Saarma M, Goldman A (2004) The structure of GFRα1 domain 3 reveals new insights into GDNF binding and RET activation. EMBO J 23: 1452-1462.

Li Y, Jiang YC, Owyang C (1998) Central CGRP inhibits pancreatic enzyme secretion by modulation of vagal parasympathetic outflow. Am J Physiol 275: G957-G963.

Lin HY, Harris TL, Flannery MS, Aruffo A, Kaji EH, Gorn A, Kolakowski LF, Jr., Lodish HF, Goldring SR (1991) Expression cloning of an adenylate cyclase-coupled calcitonin receptor. Science 254: 1022-1024.

Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260: 1130-1132.

Lindahl M, Timmusk T, Rossi J, Saarma M, Airaksinen MS (2000) Expression and alternative splicing of mouse *Gfra4* suggest roles in endocrine cell development. Mol Cell Neurosci 15: 522-533.

Lindahl M, Poteryaev D, Yu L, Arumäe U, Timmusk T, Bongarzone I, Aiello A, Pierotti MA, Airaksinen MS, Saarma M (2001) Human glial cell line-derived

neurotrophic factor receptor α4 is the receptor for persephin and is predominantly expressed in normal and malignant thyroid medullary cells. J Biol Chem 276: 9344-9351.

Lindahl M (2004) Non-neuronal roles for GDNF and novel GDNF family receptors. Dissertationes Biocentri Viikki, Universitatis Helsingiensis 21/2004

Liu KP, Russo AF, Hsiung SC, Adlersberg M, Franke TF, Gershon MD, Tamir H (2003) Calcium receptor-induced serotonin secretion by parafollicular cells: role of phosphatidylinositol 3-kinase-dependent signal transduction pathways. J Neurosci 23: 2049-2057.

Llewellyn-Smith IJ, Costa M, Furness JB, Bornstein JC (1993) Structure of the tertiary component of the myenteric plexus in the guinea-pig small intestine. Cell Tissue Res 272: 509-516.

Lo L, Tiveron MC, Anderson DJ (1998) MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. Development 125: 609-620.

Lore F, Di Cairano G, Talidis F (2000) Unilateral renal agenesis in a family with medullary thyroid carcinoma. N Engl J Med 20;342: 1218-1219.

Luukko K, Suvanto P, Saarma M, Thesleff I (1997) Expression of GDNF and its receptors in developing tooth is developmentally regulated and suggests multiple roles in innervation and organogenesis. Dev Dyn 210: 463-471.

Luukko K, Saarma M, Thesleff I (1998) Neurturin mRNA expression suggests roles in trigeminal innervation of the first branchial arch and in tooth formation. Dev Dyn 213: 207-219.

Ma Q, Fode C, Guillemot F, Anderson DJ (1999) Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. Genes Dev 13: 1717-1728.

Manie S, Santoro M, Fusco A, Billaud M (2001) The RET receptor: function in development and dysfunction in congenital malformation. Trends Genet 17: 580-589.

Maquat LE (2004) Nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. Nat Rev Mol Cell Biol 5: 89-99.

Marcos C, Pachnis V (1996) The effect of the ret- mutation on the normal development of the central and parasympathetic nervous systems. Int J Dev Biol Suppl 1: 137S-138S.

Maro GS, Vermeren M, Voiculescu O, Melton L, Cohen J, Charnay P, Topilko P (2004) Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat Neurosci 7: 930-938.

Matsui M, Motomura D, Karasawa H, Fujikawa T, Jiang J, Komiya Y, Takahashi S, Taketo MM (2000) Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M_3 subtype. PNAS 97:9579-9584.

Matsuo A, Nakamura S, Akiguchi I (2000) Immunohistochemical localization of glial cell line-derived neurotrophic factor family receptor α-1 in the rat brain: confirmation of expression in various neuronal systems. Brain Res 859:57-71.

McGaraughty S, Wismer CT, Zhu CZ, Mikusa J, Honore P, Chu KL, Lee CH, Faltynek CR, Jarvis MF (2003) Effects of A-317491, a novel and selective P2X3/P2X2/3 receptor antagonist, on neuropathic, inflammatory and chemogenic nociception following intrathecal and intraplantar administration. Br J Pharmacol 140: 1381-1388.

McMahon S, Bennett DLH, Bevan S (2006) Inflammatory mediators and modulators of pain. Wall and Melzack's textbook of pain. McMahon S and Koltzenburg M (eds.), 5th edition, Elsevier, pp. 49-72

Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287: 1489-1493.

Messer CJ, Eisch AJ, Carlezon WAJ, Whisler K, Shen L, Wolf DH, Westphal H, Collins F, Russell DS, Nestler EJ (2000) Role for GDNF in biochemical and behavioral adaptations to drugs of abuse. Neuron 26: 247-257.

Meyer RA, Ringkamp M, Campbell JN, Srinivasa NR (2006) Peripheral mechanisms of cutaneous nociception. In Wall and Melzack's textbook of pain. McMahon S and Koltzenburg M (eds.), 5th edition, Elsevier, pp. 3-34

Milbrandt J, de Sauvage FJ, Fahrner TJ, Baloh RH, Leitner ML, Tansey MG, Lampe PA, Heuckeroth RO, Kotzbauer PT, Simburger KS, Golden JP, Davies JA, Vejsada R, Kato AC, Hynes M, Sherman D, Nishimura M, Wang LC, Vandlen R, Moffat B, Klein RD, Poulsen K, Gray C, Garces A, Johnson EM, Jr. (1998) Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron 20: 245-253.

Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. Neuron 19: 849-861.

Moore MW, Klein RD, Farinas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature 382: 76-79.

Moqrich A, Hwang SW, Earley TJ, Petrus MJ, Murray AN, Spencer KS, Andahazy M, Story GM, Patapoutian A (2005) Impaired thermosensation in mice lacking TRPV3, a heat and camphor sensor in the skin. Science 307: 1468-1472.

Morales MA, Holmberg K, Xu ZQ, Cozzari C, Hartman BK, Emson P, Goldstein M, Elfvin LG, Hokfelt T (1995) Localization of choline acetyltransferase in rat peripheral sympathetic neurons and its coexistence with nitric oxide synthase and neuropeptides. Proc Natl Acad Sci U S A 92: 11819-11823.

Mount HT, Dean DO, Alberch J, Dreyfus CF, Black IB (1995) Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells. Proc Natl Acad Sci U S A 92: 9092-9096.

Munro S (2003) Lipid rafts: elusive or illusive? Cell 115: 377-388.

Nanobashvili A, Airaksinen MS, Kokaia M, Rossi J, Asztely F, Olofsdotter K, Mohapel P, Saarma M, Lindvall O, Kokaia Z (2000) Development and persistence of kindling epilepsy are impaired in mice lacking glial cell line-derived neurotrophic factor family receptor α2. Proc Natl Acad Sci U S A 97: 12312-12317.

Natarajan D, Marcos-Gutierrez C, Pachnis V, de Graaff E (2002) Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development 129: 5151-5160.

Navarro X, Verdu E, Wendelscafer-Crabb G, Kennedy WR (1995) Innervation of cutaneous structures in the mouse hind paw: a confocal microscopy immunohistochemical study. J Neurosci Res 41: 111-120.

Naveilhan P, Baudet C, Mikaels A, Shen L, Westphal H, Ernfors P (1998) Expression and regulation of GFRalpha3, a glial cell line-derived neurotrophic factor family receptor. Proc Natl Acad Sci U S A 95: 1295-1300.

Newgreen D, Young HM (2002a) Enteric nervous system: development and developmental disturbances--part 1. Pediatr Dev Pathol 5: 224-247.

Newgreen D, Young HM (2002b) Enteric nervous system: development and developmental disturbances--part 2. Pediatr Dev Pathol 5: 329-349.

Nguyen QT, Parsadanian AS, Snider WD, Lichtman JW (1998) Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. Science 279: 1725-1729.

Nicholson GC, Moseley JM, Sexton PM, Mendelsohn FA, Martin TJ (1986) Abundant calcitonin receptors in isolated rat osteoclasts. Biochemical and autoradiographic characterization. J Clin Invest 78: 355-360.

Nishino J, Mochida K, Ohfuji Y, Shimazaki T, Meno C, Ohishi S, Matsuda Y, Fujii H, Saijoh Y, Hamada H (1999) GFRα3, a component of the artemin receptor, is required for migration and survival of the superior cervical ganglion. Neuron 23: 725-736.

Nosrat CA, Tomac A, Hoffer BJ, Olson L (1997) Cellular and developmental patterns of expression of Ret and glial cell line-derived neurotrophic factor receptor alpha mRNAs. Exp Brain Res 115: 410-422.

Nunez EA, Gershon MD (1972) Synthesis and storage of serotonin by parafollicular (C) cells of the thyroid gland of active, prehibernating and hibernating bats. Endocrinology 90: 1008-1024.

Oaklander AL (2001) The density of remaining nerve endings in human skin with and without postherpetic neuralgia after shingles. Pain 92: 139-145.

Oo TF, Kholodilov N, Burke RE (2003) Regulation of natural cell death in dopaminergic neurons of the substantia nigra by striatal glial cell line-derived neurotrophic factor in vivo. J Neurosci 23: 5141-5148.

Oppenheim RW, Houenou LJ, Parsadanian AS, Prevette D, Snider WD, Shen L (2000) Glial cell line-derived neurotrophic factor and developing mammalian motoneurons: regulation of programmed cell death among motoneuron subtypes. J Neurosci 20: 5001-5011.

Orozco OE, Walus L, Sah DW, Pepinsky RB, Sanicola M (2001) GFRalpha3 is expressed predominantly in nociceptive sensory neurons. Eur J Neurosci 13: 2177-2182.

Owyang C (1996) Physiological mechanisms of cholecystokinin action on pancreatic secretion. Am J Physiol 271: G1-G7.

Pachnis V, Mankoo B, Costantini F (1993) Expression of the c-ret proto-oncogene during mouse embryogenesis. Development 119: 1005-1017.

Paratcha G, Ledda F, Baars L, Coulpier M, Besset V, Anders J, Scott R, Ibanez CF (2001) Released GFRα1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. Neuron 29: 171-184.

Paratcha G, Ledda F, Ibanez CF (2003) The Neural Cell Adhesion Molecule NCAM Is an Alternative Signaling Receptor for GDNF Family Ligands. Cell 113: 867-879.

Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR (1987) Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res 2: 595-610.

Parisi MA, Kapur RP (2000) Genetics of Hirschsprung disease. Curr Opin Pediatr 12: 610-617.

Patapoutian A, Peier AM, Story GM, Viswanath V (2003) ThermoTRP channels and beyond: mechanisms of temperature sensation. Nat Rev Neurosci 4: 529-539.

Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1999) The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature 399: 366-370.

Paveliev M, Airaksinen MS, Saarma M (2004) GDNF family ligands activate multiple events during axonal growth in mature sensory neurons. Mol Cell Neurosci 25: 453-459.

Periquet MI, Novak V, Collins MP, Nagaraja HN, Erdem S, Nash SM, Freimer ML, Sahenk Z, Kissel JT, Mendell JR (1999) Painful sensory neuropathy: prospective evaluation using skin biopsy. Neurology 53: 1641-1647.

Peters RJ, Osinski MA, Hongo JA, Bennett GL, Okragly AJ, Haak-Frendscho M, Epstein ML (1998) GDNF is abundant in the adult rat gut. 70(1-2):115-122.

Peterziel H, Unsicker K, Krieglstein K (2002) TGFbeta induces GDNF responsiveness in neurons by recruitment of GFRalpha1 to the plasma membrane. J Cell Biol 159: 157-167.

Pezeshki G, Franke B, Engele J (2001) Evidence for a ligand-specific signaling through GFRα-1, but not GFRα-2, in the absence of Ret. J Neurosci Res 66: 390-395.

Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 382: 73-76.

Pitcher GM, Henry JL (2002) Second phase of formalin-induced excitation of spinal dorsal horn neurons in spinalized rats is reversed by sciatic nerve block. Eur J Neurosci 15: 1509-1515.

Popsueva A, Poteryaev D, Arighi E, Meng X, Angers-Loustau A, Kaplan D, Saarma M, Sariola H (2003) GDNF promotes tubulogenesis of GFRα1-expressing MDCK cells by Src-mediated phosphorylation of Met receptor tyrosine kinase. J Cell Biol 161: 119-129.

Poteryaev D, Titievsky A, Sun YF, Thomas-Crusells J, Lindahl M, Billaud M, Arumae U, Saarma M (1999) GDNF triggers a novel Ret-independent Src kinase family-coupled signaling via a GPI-linked GDNF receptor α1. FEBS Lett 463: 63-66.

Pozas E, Ibanez CF (2005) GDNF and GFRα1 promote differentiation and tangential migration of cortical GABAergic neurons. Neuron 45:701-713.

Puig S, Sorkin LS (1995) Formalin-evoked activity in identified primary afferent fibers: systemic lidocaine suppresses phase-2 activity. Pain 64: 345-355.

Quartu M, Pina Serra M, Manca A, Mascia F, Follesa P, Del Fiacco M (2005) Neurturin, persephin and artemin in the human pre- and full-term newborn and adult hippocampus and facia dentata. Brain Res 1041:157-166.

Quartu M, Pina Serra M, Mascia F, Boi M, Lai ML, Spano A, Del Fiacco M (2006) GDNF family ligand receptor components Ret and GFRalpha-1 in the human trigeminal ganglion and sensory nuclei. Brain Res 69:393-403.

Ramer MS, Priestley JV, McMahon SB (2000) Functional regeneration of sensory axons into the adult spinal cord. Nature 403: 312-316.

Randall D, Burggren W, French K. (1997) Eckert animal physiology, mechanisms and adaptations. 4th edition, Freeman, pp. 273-350

Ribchester RR, Thomson D, Haddow LJ, Ushkaryov YA (1998) Enhancement of spontaneous transmitter release at neonatal mouse neuromuscular junctions by the glial cell line-derived neurotrophic factor (GDNF). J Physiol 512: 635-641.

Rogers RC, McTigue DM, Hermann GE (1996) Vagal control of digestion: modulation by central neural and peripheral endocrine factors. Neurosci Biobehav Rev 20: 57-66.

Rosenfeld MG, Mermod JJ, Amara SG, Swanson LW, Sawchenko PE, Rivier J, Vale WW, Evans RM (1983) Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature 304: 129-135.

Rossi J, Luukko K, Poteryaev D, Laurikainen A, Sun Y-F, Laakso T, Eerikäinen S, Tuominen R, Lakso M, Rauvala H, Arumäe U, Saarma M, Airaksinen MS (1999) Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFRα2, a functional neurturin receptor. Neuron 22: 243-252.

Rossi J, Tomac A, Saarma M, Airaksinen MS (2000) Distinct roles for GFRα1 and GFRα2 signalling in different cranial parasympathetic ganglia in vivo. Eur J Neurosci 12: 3944-3952.

Rossi J, Santamaki P, Airaksinen MS, Herzig KH (2005) Parasympathetic innervation and function of endocrine pancreas requires the glial cell line-derived factor family receptor a2 (GFRα2). Diabetes 54: 1324-1330.

Sainio K, Suvanto P, Davies J, Wartiovaara J, Wartiovaara K, Saarma M, Arumae U, Meng X, Lindahl M, Pachnis V, Sariola H (1997) Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium. Development 124: 4077-4087.

Sanchez MP, Silos-Santiago I, Frisen J, He B, Lira SA, Barbacid M (1996) Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature 382: 70-73.

Sanes DH, Reh TA, Harris WA (2006) Development of the nervous system. 2nd edition, Elsevier, pp. 50-66

Sang Q, Williamson S, Young HM (1997) Projections of chemically identified myenteric neurons of the small and large intestine of the mouse. J Anat 190 (Pt 2): 209-222.

Sang Q, Young HM (1998) The identification and chemical coding of cholinergic neurons in the small and large intestine of the mouse. Anat Rec 251: 185-199.

Sanicola M, Hession C, Worley D, Carmillo P, Ehrenfels C, Walus L, Robinson S, Jaworski G, Wei H, Tizard R, Whitty A, Pepinsky RB, Cate RL (1997) Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. Proc Natl Acad Sci U S A 94: 6238-6243.

Santisteban P, Bernal J (2005) Thyroid development and effect on the nervous system. Rev Endocr Metab Disord 6: 217-228.

Sariola H, Sainio K, Arumae U, Saarma M (1994) Neurotrophins and ciliary neurotrophic factor: their biology and pathology. Ann Med 26: 355-363.

Sariola H (2001) The neurotrophic factors in non-neuronal tissues. Cell Mol Life Sci 58: 1061-1066.

Sariola H, Saarma M (2003) Novel functions and signalling pathways for GDNF. J Cell Sci 116: 3855-3862.

Schafer MK, Schutz B, Weihe E, Eiden LE (1997) Target-independent cholinergic differentiation in the rat sympathetic nervous system. Proc Natl Acad Sci U S A 94: 4149-4154.

Schober A, Hertel R, Arumae U, Farkas L, Jaszai J, Krieglstein K, Saarma M, Unsicker K (1999) Glial cell line-derived neurotrophic factor rescues target-deprived sympathetic spinal cord neurons but requires transforming growth factor-beta as cofactor in vivo. J Neurosci JID - 8102140 19: 2008-2015.

Schober A, Arumae U, Saarma M, Unsicker K (2000) Expression of GFR alpha-1, GFR alpha-2, and c-Ret mRNAs in rat adrenal gland. J Neurocytol 29: 209-213.

Schotzinger RJ, Landis SC (1988) Cholinergic phenotype developed by noradrenergic sympathetic neurons after innervation of a novel cholinergic target in vivo. Nature 335: 637-639.

Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature 367: 380-383.

Schueler-Furman O, Glick E, Segovia J, Linial M (2006) Is GAS1 a co-receptor for the GDNF family of ligands? Trends Pharmacol Sci 27: 72-77.

Schwitzgebel VM (2001) Programming of the pancreas. Mol Cell Endocrinol 185: 99-108.

Shen L, Pichel JG, Mayeli T, Sariola H, Lu B, Westphal H (2002) Gdnf haploinsufficiency causes Hirschsprung-like intestinal obstruction and early-onset lethality in mice. Am J Hum Genet 70: 435-447.

Shepherd IT, Pietsch J, Elworthy S, Kelsh RN, Raible DW (2004) Roles for GFRalpha1 receptors in zebrafish enteric nervous system development. Development 131: 241-249.

Shimizu I, Iida T, Guan Y, Zhao C, Raja SN, Jarvis MF, Cockayne DA, Caterina MJ (2005) Enhanced thermal avoidance in mice lacking the ATP receptor P2X(3). Pain Jul;116(1-2):96-108

Silos-Santiago I, Molliver DC, Ozaki S, Smeyne RJ, Fagan AM, Barbacid M, Snider WD (1995) Non-TrkA-expressing small DRG neurons are lost in TrkA deficient mice. J Neurosci 15: 5929-5942.

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387: 569-72.

Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1: 31-39.

Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. Neuron 20: 629-632.

Sofroniew MV, Howe CL, Mobley WC (2001) Nerve growth factor signaling, neuroprotection, and neural repair. Annu Rev Neurosci 24:1217-1281.

Song XJ, Li DQ, Farley W, Luo LH, Heuckeroth RO, Milbrandt J, Pflugfelder SC (2003) Neurturin-deficient mice develop dry eye and keratoconjunctivitis sicca. Invest Ophthalmol Vis Sci 44: 4223-4229.

Souslova V, Cesare P, Ding Y, Akopian AN, Stanfa L, Suzuki R, Carpenter K, Dickenson A, Boyce S, Hill R, Nebenuis-Oosthuizen D, Smith AJ, Kidd EJ, Wood JN (2000) Warm-coding deficits and aberrant inflammatory pain in mice lacking P2X3 receptors. Nature 407: 1015-1017.

Stanke M, Geissen M, Gotz R, Ernsberger U, Rohrer H (2000) The early expression of VAChT and VIP in mouse sympathetic ganglia is not induced by cytokines acting through LIFRbeta or CNTFRalpha. Mech Dev 91: 91-96.

Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell 112: 819-829.

Stucky CL, Rossi J, Airaksinen MS, Lewin GR (2002) GFR alpha2/neurturin signalling regulates noxious heat transduction in isolectin B4-binding mouse sensory neurons. J Physiol 545: 43-50.

Suvanto P, Hiltunen JO, Arumäe U, Moshnyakov M, Sariola H, Sainio K, Saarma M (1996) Localization of glial cell line-derived neurotrophic factor (GDNF) mRNA in embryonic rat by in situ hybridization. Eur J Neurosci 8: 816-822.

Suzuki K, Lavaroni S, Mori A, Okajima F, Kimura S, Katoh R, Kawaoi A, Kohn LD (1998) Thyroid transcription factor 1 is calcium modulated and coordinately regulates genes involved in calcium homeostasis in C cells. Mol Cell Biol 18: 7410-7422.

Tahira T, Ishizaka Y, Itoh F, Sugimura T, Nagao M (1990) Characterization of ret proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. Oncogene 5: 97-102.

Takahashi M, Ritz J, Cooper GM (1985) Activation of a novel human transforming gene, ret, by DNA rearrangement. Cell 42: 581-588.

Takahashi M, Buma Y, Iwamoto T, Inaguma Y, Ikeda H, Hiai H (1988) Cloning and expression of the ret proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. Oncogene 3: 571-578.

Takahashi M, Buma Y, Hiai H (1989) Isolation of ret proto-oncogene cDNA with an amino-terminal signal sequence. Oncogene 4: 805-806.

Takahashi M (2001) The GDNF/RET signaling pathway and human diseases. Cytokine Growth Factor Rev 12: 361-373.

Takaya K, Yoshimasa T, Arai H, Tamura N, Miyamoto Y, Itoh H, Nakao K (1996) Expression of the RET proto-oncogene in normal human tissues, pheochromocytomas, and other tumors of neural crest origin. J Mol Med 74: 617-621.

Tansey MG, Baloh RH, Milbrandt J, Johnson EM, Jr. (2000) GFRα-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. Neuron 25: 611-623.

Taylor BK, Peterson MA, Basbaum AI (1995) Persistent cardiovascular and behavioral nociceptive responses to subcutaneous formalin require peripheral nerve input. J Neurosci 15: 7575-7584.

Thompson J, Doxakis E, Pinon LGP, Strachan P, Buj-Bello A, Wyatt S, Buchman VL, Davies AM (1998) GFRα-4, a new GDNF family receptor. Mol Cell Neurosci 11:117-126.

Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K (1992) The formalin test: an evaluation of the method. Pain 51: 5-17.

Tomac AC, Agulnick AD, Haughey N, Chang CF, Zhang Y, Backman C, Morales M, Mattson MP, Wang Y, Westphal H, Hoffer BJ (2002) Effects of cerebral ischemia in mice deficient in Persephin. Proc Natl Acad Sci U S A 99: 9521-9526.

Tominaga M, Caterina MJ (2004) Thermosensation and pain. J Neurobiol 61: 3-12.

Treanor JJ, Goodman L, de Sauvage F, Stone DM, Poulsen KT, Beck CD, Gray C, Armanini MP, Pollock RA, Hefti F, Phillips HS, Goddard A, Moore MW, Buj-Bello A, Davies AM, Asai N, Takahashi M, Vandlen R, Henderson CE, Rosenthal A (1996) Characterization of a multicomponent receptor for GDNF. Nature 382: 80-83.

Trupp M, Ryden M, Jornvall H, Funakoshi H, Timmusk T, Arenas E, Ibáñez CF (1995) Peripheral expression and biological activities of GDNF, a new

neurotrophic factor for avian and mammalian peripheral neurons. J Cell Biol 130: 137-148.

Trupp M, Belluardo N, Funakoshi H, Ibáñez CF (1997) Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. J Neurosci 17: 3554-3567.

Trupp M, Scott R, Whittemore SR, Ibáñez CF (1999) Ret-dependent and -independent mechanisms of glial cell line-derived neurotrophic factor signaling in neuronal cells. J Biol Chem 274: 20885-20894.

Tsuda M, Ueno S, Inoue K (1999) Evidence for the involvement of spinal endogenous ATP and P2X receptors in nociceptive responses caused by formalin and capsaicin in mice. Br J Pharmacol 128: 1497-1504.

Tsui-Pierchala BA, Ahrens RC, Crowder RJ, Milbrandt J, Johnson EMJ (2002a) The long and short isoforms of Ret function as independent signaling complexes. J Biol Chem 277(37):34618-34625.

Tsui-Pierchala BA, Milbrandt J, Johnson EM (2002b) NGF utilizes c-Ret via a novel GFL-independent, inter-RTK signaling mechanism to maintain the trophic status of mature sympathetic neurons. Neuron 33: 261-273.

Tsuzuki T, Takahashi M, Asai N, Iwashita T, Matsuyama M, Asai J (1995) Spatial and temporal expression of the ret proto-oncogene product in embryonic, infant and adult rat tissues. Oncogene 10: 191-198.

Unsicker K, Huber K, Schutz G, Kalcheim C (2005) The chromaffin cell and its development. Neurochem Res 30: 921-925.

Vanhorne JB, Andrew SD, Harrison KJ, Taylor SA, Thomas B, McDonald TJ, Ainsworth PJ, Mulligan LM (2005) A model for GFR alpha 4 function and a potential modifying role in multiple endocrine neoplasia 2. Oncogene 24: 1091-1097.

Vellani V, Zachrisson O, McNaughton PA (2004) Functional bradykinin B1 receptors are expressed in nociceptive neurones and are upregulated by the neurotrophin GDNF. J Physiol 560: 391-401.

Voikar V, Rossi J, Rauvala H, Airaksinen MS (2004) Impaired behavioural flexibility and memory in mice lacking GDNF family receptor alpha2. Eur J Neurosci 20: 308-312.

Vulchanova L, Riedl MS, Shuster SJ, Stone LS, Hargreaves KM, Buell G, Surprenant A, North RA, Elde R (1998) P2X3 is expressed by DRG neurons that terminate in inner lamina II. Eur J Neurosci 10: 3470-3478.

Wanigasekara Y, Airaksinen MS, Heuckeroth RO, Milbrandt J, Keast JR (2004) Neurturin signalling via GFRalpha2 is essential for innervation of glandular but not muscle targets of sacral parasympathetic ganglion neurons. Mol Cell Neurosci 25: 288-300.

Warner G, Sharief MK, Anand P (2002) Small sensory fibre dysfunction in selective cholinergic dysautonomia. Eur J Neurol 9: 109.

Widenfalk J, Nosrat C, Tomac A, Westphal H, Hoffer B, Olson L (1997) Neurturin and glial cell line-derived neurotrophic factor receptor-beta (GDNFR-beta), novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. J Neurosci 17: 8506-8519.

Widenfalk J, Tomac A, Lindqvist E, Hoffer B, Olson L (1998) GFRalpha-3, a protein related to GFRalpha-1, is expressed in developing peripheral neurons and ensheathing cells. Eur J Neurosci 10: 1508-1517.

Williams LR, Inouye G, Cummins V, Pelleymounter MA (1996) Glial cell line-derived neurotrophic factor sustains axotomized basal forebrain cholinergic neurons in vivo: dose-response comparison to nerve growth factor and brain-derived neurotrophic factor. J Pharmacol Exp Ther 277: 1140-1151.

Wilson AJ, Llewellyn-Smith IJ, Furness JB, Costa M (1987) The source of the nerve fibres forming the deep muscular and circular muscle plexuses in the small intestine of the guinea-pig. Cell Tissue Res 247: 497-504.

Woodbury CJ, Zwick M, Wang S, Lawson JJ, Caterina MJ, Koltzenburg M, Albers KM, Koerber HR, Davis BM (2004) Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. J Neurosci 24: 6410-6415.

Woolf CJ, Costigan M (1999) Transcriptional and posttranslational plasticity and the generation of inflammatory pain. Proc Natl Acad Sci U S A 96: 7723-7730.

Worley DS, Pisano JM, Choi ED, Walus L, Hession CA, Cate RL, Sanicola M, Birren SJ (2000) Developmental regulation of GDNF response and receptor expression in the enteric nervous system. Development 127: 4383-4393.

Xian CJ, Huang BR, Zhou XF (1999) Distribution of neurturin mRNA and immunoreactivity in the peripheral tissues of adult rats. Brain Res 835: 247-258.

Yamada M, Miyakawa T, Duttaroy A, Yamanaka A, Moriguchi T, Makita R, Ogawa M, Chou CJ, Xia B, Crawley JN, Felder CC, Deng C-X, Wess J (2001) Mice lacking the M3 muscarinic acetylcholine receptor are hypophagic and lean. Nature 410:207-212.

Yang F, Feng L, Zheng F, Johnson SW, Du J, Shen L, Wu CC, Lu B (2001) GDNF acutely modulates excitability and A-type K+ channels in midbrain dopaminergic neurons. Nat Neurosci 4: 1071-1078.

Yang J, Lindahl M, Lindholm P, Virtanen H, Coffey E, Runeberg-Roos P, Saarma M (2004) PSPN/GFRalpha4 has a significantly weaker capacity than GDNF/GFRalpha1 to recruit RET to rafts, but promotes neuronal survival and neurite outgrowth. FEBS Lett 569: 267-271.

Yang, J., Rauvala, H., and Saarma, M (2005) A novel transmembrane receptor GFRa4 isoform silences persephin-mediated RET signaling, differentiation, and neuronal survival. Program No.599.8.2005 Abstract Viewer/Itinerary Planner.Washington, DC: Society for Neuroscience, 2005.Online.

Young HM, Hearn CJ, Ciampoli D, Southwell BR, Brunet JF, Newgreen DF (1998) A single rostrocaudal colonization of the rodent intestine by enteric neuron precursors is revealed by the expression of Phox2b, Ret, and p75 and by explants grown under the kidney capsule or in organ culture. Dev Biol 202: 67-84.

Zaidi M, Inzerillo AM, Moonga BS, Bevis PJ, Huang CL (2002) Forty years of calcitonin--where are we now? A tribute to the work of Iain Macintyre, FRS. Bone 30: 655-663.

Zwick M, Davis BM, Woodbury CJ, Burkett JN, Koerber HR, Simpson JF, Albers KM (2002) Glial cell line-derived neurotrophic factor is a survival factor for isolectin B4-positive, but not vanilloid receptor 1-positive, neurons in the mouse. J Neurosci 22: 4057-4065.

Zylka MJ, Rice FL, Anderson DJ (2005) Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. Neuron 45: 17-25.

Åkerud P, Alberch J, Eketjäll S, Wagner J, Arenas E (1999) Differential effects of glial cell line-derived neurotrophic factor and neurturin on developing and adult substantia nigra dopaminergic neurons. J Neurochem 73: 70-78.

Åkerud P, Canals JM, Snyder EY, Arenas E (2001) Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's disease. J Neurosci 21: 8108-8118.

Åkerud P, Holm PC, Castelo-Branco G, Sousa K, Rodriguez FJ, Arenas E (2002) Persephin-overexpressing neural stem cells regulate the function of nigral dopaminergic neurons and prevent their degeneration in a model of Parkinson's disease. Mol Cell Neurosci 21: 205-222.