Novel functions and signalling pathways for GDNF

Hannu Sariola[1,*] and Mart Saarma[2]

[1]Institute of Biomedicine, University of Helsinki, PO Box 63 (Haartmaninkatu 8), FIN-00014 and HUCH Laboratory Diagnostics, PO Box 400, Helsinki University Central Hospital, FIN-00029, Finland
[2]Institute of Biotechnology, University of Helsinki, PO Box 56 (Viikinkaari 9), University of Helsinki, FIN-00014, Finland
*Author for correspondence (e-mail: hannu.sariola@helsinki.fi)

Journal of Cell Science 116, 3855-3862 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00786

Summary

Glial-cell-line-derived neurotrophic factor (GDNF) was originally identified as a survival factor for midbrain dopaminergic neurons. GDNF and related ligands, neurturin (NRTN), artemin (ARTN) and persephin (PSPN), maintain several neuronal populations in the central nervous systems, including midbrain dopamine neurons and motoneurons. In addition, GDNF, NRTN and ARTN support the survival and regulate the differentiation of many peripheral neurons, including sympathetic, parasympathetic, sensory and enteric neurons. GDNF has further critical roles outside the nervous system in the regulation of kidney morphogenesis and spermatogenesis. GDNF family ligands bind to specific GDNF family receptor α (GFRα) proteins, all of which form receptor complexes and signal through the RET receptor tyrosine kinase. The biology of GDNF signalling is much more complex than originally assumed. The neurotrophic effect of GDNF, except in motoneurons, requires the presence of transforming growth factor β, which activates the transport of GFRα1 to the cell membrane. GDNF can also signal RET independently through GFR1α. Upon ligand binding, GDNF in complex with GFRα1 may interact with heparan sulphate glycosaminoglycans to activate the Met receptor tyrosine kinase through cytoplasmic Src-family kinases. GDNF family ligands also signal through the neural cell adhesion molecule NCAM. In cells lacking RET, GDNF binds with high affinity to the NCAM and GFRα1 complex, which activates Fyn and FAK.

Key words: Glial-cell-line-derived neurotrophic factor, RET receptor tyrosine kinase, Met receptor tyrosine kinase, GDNF family receptor α, NCAM, Neuronal survival, Kidney morphogenesis, Spermatogenesis

Introduction

Neurotrophic factors regulate many critical aspects of the ontogeny of neurons, such as the number of neurons in a given population, neurite branching and synaptogenesis, adult synaptic plasticity and maturation of electrophysiological properties. Neurotrophic factors include neurotrophins, neurokines and glial-cell-line-derived neurotrophic factor (GDNF) family ligands (GFLs). GDNF was purified and characterized in 1993 as a growth factor promoting the survival of the embryonic dopaminergic neurons of the midbrain, i.e. those neurons that degenerate in Parkinson disease (Lin et al., 1993). Subsequently, it was shown that GDNF is also a very potent trophic factor for spinal motoneurons (Henderson et al., 1994) and central noradrenergic neurons (Arenas et al., 1995). Therefore, this trophic factor raised great expectations as a potential therapeutic agent for the treatment of neurodegenerative diseases. In animal models of Parkinson disease, GDNF rescues the neurotoxin-induced death of dopamine neurons and stimulates functional recovery (Grondin and Gash, 1998). However, in early clinical trials, in which GDNF was delivered into the lateral ventricles of patients with Parkinson disease, the growth factor was ineffective and caused severe side-effects. A recent clinical trial indicates that, when the GDNF is infused directly in putamen, it is an effective treatment for Parkinson disease and does not have significant side-effects (Gill et al., 2003).

GDNF and the related GFLs artemin (ARTN), neurturin (NRTN) and persephin (PSPN) support several neuronal populations in the central nervous system, including midbrain dopamine neurons and motoneurons. In addition, GDNF, NRTN and ARTN promote the survival and regulate the differentiation of many peripheral neurons, such as sympathetic, parasympathetic, sensory and enteric neurons (reviewed by Airaksinen et al., 1999; Manié et al., 2001; Airaksinen and Saarma, 2002).

GDNF has several roles outside the nervous system. It functions as a morphogen in kidney development and regulates spermatogonial differentiation. In the embryonic kidney, GDNF acts as a mesenchyme-derived signal promoting ureteric branching. In the testis, the GDNF dosage controls the cell fate decision of undifferentiated spermatogonia (reviewed by Saarma and Sariola, 1999; Airaksinen and Saarma, 2002).

The cellular responses to GFLs are mediated by a multicomponent receptor complex consisting of RET receptor tyrosine kinase and a glycosyl phosphatidylinositol (GPI)-linked ligand-binding subunit known as GDNF family receptor α (GFRα). Several recent in vitro findings demonstrate that GFLs also signal independently of RET, and in particular through NCAM. Here we focus on the new receptors for GDNF and the functional implications.

GDNF receptors

All GFLs share the receptor tyrosine kinase RET as their common signalling receptor. The ligand-binding specificity of GFLs is determined by GFRα proteins that have unique

binding affinities for each GFL. GDNF, NRTN, ARTN and PSPN specifically bind to GFRα1, GFRα2, GFRα3 and GFRα4, respectively. The GFLs first form a high-affinity complex with one of the four GFRα proteins. The complex, containing GFL and GFRα homodimers, then brings two molecules of RET together, triggering transphosphorylation of specific tyrosine residues in their tyrosine kinase domains and intracellular signalling (Airaksinen and Saarma, 2002) (Fig. 1).

RET activates several intracellular signalling cascades, which regulate cell survival, differentiation, proliferation, migration, chemotaxis, branching morphogenesis, neurite outgrowth and synaptic plasticity. The MAP kinase pathway may be involved in ureteric branching during nephrogenesis (Fisher et al., 2001) and neurite outgrowth in the nervous system, but it also contributes to neuronal survival (Kaplan and Miller, 2000). The phosphoinositide 3-kinase (PI3K) pathway is crucial for both neuronal survival and neurite outgrowth. The phospholipase Cγ (PLC-γ) pathway regulates the intracellular level of Ca^{2+} ions by increasing the level of inositol (1,4,5)-trisphosphate. GDNF signalling also employs Src-family kinases, which elicite neurite outgrowth, neuronal survival and ureteric branching (Airaksinen and Saarma, 2002). In most cases tyrosine residues Tyr905, Tyr1015, Tyr1062 and Tyr1096 of RET are phosphorylated, but after the elevation of cyclic AMP levels, Ser696 is also phosphorylated. Protein kinase A (PKA)-dependent Ser696 phosphorylation is important for GDNF-induced Rac activation and lamellipodia formation (Fukuda et al., 2002). RET contains additional tyrosine residues that are phosphorylated upon GFL binding (Tyr687, Tyr826 and Tyr 1029), but the role of these in GFL signalling remains obscure.

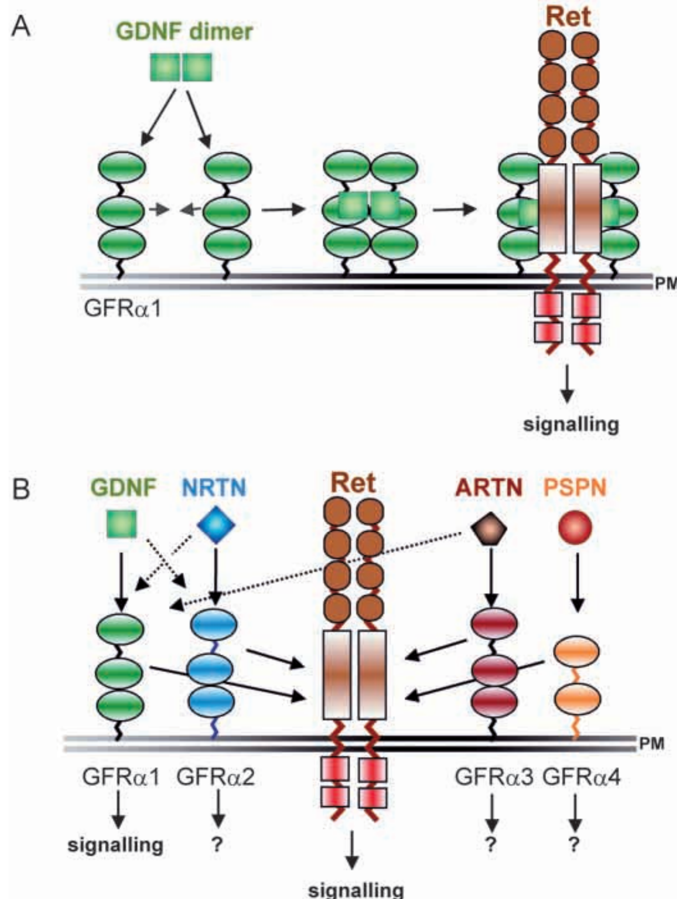

Fig. 1. GDNF-family ligand interaction with their receptors. (A) A dimer of GDNF brings together two molecules of GFRα1. This complex dimerizes two molecules of Ret leading to transphophorylation of their tyrosine kinase domains. (B) All GFLs activate RET tyrosine kinase via different GFRα receptors. Solid arrows indicate the preferred functional ligand-receptor interactions, whereas dotted arrows indicate putative crosstalk. GFRα proteins are attached to the plasma membrane through a GPI-anchor and consist of three (GFRα4 has only two) globular cysteine-rich domains joined together by adapter sequences. Ca^{2+} ions bound to one of the four extracellular cadherin-like domains of RET are needed for its activation by GFLs.

RET activation affects different downstream targets inside and outside lipid rafts (the dynamic assemblies of cholesterol and sphingolipids scattered within the disordered phase of the lipid bilayer). Lipid rafts are proposed to serve as essential signalling compartments in the cell membrane, and are important for cell adhesion, axon guidance and synaptic transmission. GPI-anchored proteins, certain transmembrane proteins, doubly acylated proteins, and cholesterol-linked and palmitoylated proteins are enriched in the rafts. However, the protein motifs responsible for their targeting to lipid rafts are largely unknown. The GFRα proteins, by the virtue of their GPI anchors, also localize to lipid rafts. Inactive RET is outside rafts, and only upon GDNF stimulation does GFRα1 recruit RET into lipid rafts; the mechanism is unknown. Soluble GFRα1 also targets RET to lipid rafts (reviewed by Paratcha and Ibañéz, 2002; Tsui-Pierchala et al., 2002a). Moreover, it prolongs GDNF-mediated activation of cyclin-dependent kinase 5 (CDK5) and acts as an attractive guidance signal for axons (Ledda et al., 2002). Activated RET is preferentially associated with the adaptor SHC outside rafts, and with FGF receptor substrate 2 (FRS2) in rafts (Paratcha and Ibañéz, 2002). These data suggest that differences in GDNF signalling through RET within and outside the rafts could lead to dramatically different cellular responses.

RET is alternatively spliced, producing at least two isoforms, RET9 and RET51, which differ only in their C-termini. Recent evidence suggests that RET9 and RET51 do not associate with each other. Furthermore, RET51- and RET9-associated signalling complexes are markedly different (Tsui-Pierchala et al., 2002b). The long isoform, RET51, associates more strongly with the ubiquitin ligase Cbl than does RET9, which leads to faster turnover of RET51. RET51 also interacts with the adaptor Crkl, producing sustained activation of Erk1 and Erk2 (R. P. Scott, Signal transduction mechanisms mediated by the GDNF family ligands and receptors, PhD thesis, Karolinska Institute, Stockholm, 2002). Mice lacking the long RET isoform seem to be normal, whereas mice lacking the short isoform have kidney abnormalities and enteric aganglionosis. Only the short RET9 isoform can rescue the phenotype of the RET-null mutation in the kidney and enteric nervous system (de Graaf et al., 2001).

GFLs belong to the transforming growth factor-β (TGF-β) superfamily, although the amino acid sequence similarity between them is low. Surprisingly, the neurotrophic effect of GDNF in vitro and in vivo, except for motoneurons, requires the presence of TGF-β (Peterziel et al., 2002). Blocking the ERK/MAPK pathway inhibits this co-operative effect, whereas

inhibition of the PI3K signalling does not. Pre-treatment of primary neuronal cultures with TGF-β confers GDNF responsiveness on the cells. This is not due to upregulation of GDNF receptor mRNA and protein but to TGF-β-induced recruitment of the GFRα1 to the plasma membrane. In the absence of TGF-β, GDNF supports neuronal survival if the soluble form of GFRα1 is present. Thus, TGF-β is involved in GFRα1 membrane translocation and in a novel way regulates GDNF signalling and neurotrophic effects. It would be of great interest to know whether TGF-β also regulates other GFRα proteins.

GDNF signalling requires glycosaminoglycans

Recent data indicate that GDNF signalling is more complicated than originally supposed. Notably, GDNF signalling requires heparan sulphate glycosaminoglycans in addition to the known receptors GFRα1 and RET (Barnett et al., 2002; Tanaka et al., 2002). Without heparan sulphate, GDNF-dependent RET phosphorylation, GDNF-induced axonal growth, and scattering of epithelial cells do not occur (Barnett et al., 2002). Thus, it is logical to assume that heparan sulphate proteoglycans, such as syndecans and glypicans, concentrate GDNF in the vicinity of GFRα proteins and RET, and play an important role in the modulation of GDNF signalling. A model, in which GDNF is locally concentrated by heparan sulphate proteoglycans at the plasma membrane is also supported by the finding that very high concentrations of GDNF activate RET even in cells depleted of surface heparin sulphates (Barnett et al., 2002). Furthermore, the mice lacking heparan sulphate 2-sulfotransferase, which is an essential enzyme in the synthesis of heparan sulphates, lack kidneys (Bullock et al., 1998). These mice have an interesting phenotype. The ureteric bud invades the metanephric mesenchyme but is unable to undergo branching morphogenesis, which is a GDNF-dependent process. Molecular analysis has shown that these mice cannot maintain GDNF and RET expression in the kidneys (Bullock et al., 1998).

GDNF can signals independently of RET

An enigma in GDNF research has been the widespread expression of GFRα proteins in many tissues without coexpression of RET. One explanation is that GDNF-GFRα signalling makes use of other receptor systems. GDNF indeed signals independently of RET through GFRα1. In RET-deficient cell lines and primary neurons, GDNF triggers Src-family kinase activation and phosphorylation of ERK/MAP kinase, PLC-γ and the transcription factor CREB, and induction of Fos (Poteryaev et al., 1999; Trupp et al., 1999). GDNF partially restores ureteric branching morphogenesis in RET-deficient mice that exhibit severe renal hypodysplasia (Popsueva et al., 2003). In MDCK cells expressing GFRα1 but not RET, GDNF stimulates branching but not chemotactic migration. Both branching and chemotaxis are promoted by GDNF in cells co-expressing RET and GFRα1, which mimics the effects of hepatocyte growth factor (HGF) signalling through the Met receptor tyrosine kinase in wild-type MDCK cells. GDNF induces Met phosphorylation in several RET-deficient but GFRα1-positive cells, as well as in cell lines co-expressing GFRα1 and RET. Met might therefore contribute to RET-independent GDNF signalling. However, GDNF does not immunoprecipitate Met, which makes a direct interaction between GDNF and Met highly improbable. In cultured neuronal and epithelial cells, Met activation is mediated by Src-family kinases. The GDNF-triggered RET-independent Src and Met activation might be modulated by heparan sulphate proteoglycans and mediated by neural cell adhesion molecule (NCAM) (see below). The GDNF-induced branching of MDCK cells requires Src activation, whereas the HGF-induced branching does not (Fig. 2A) (Popsueva et al., 2003). The in vivo

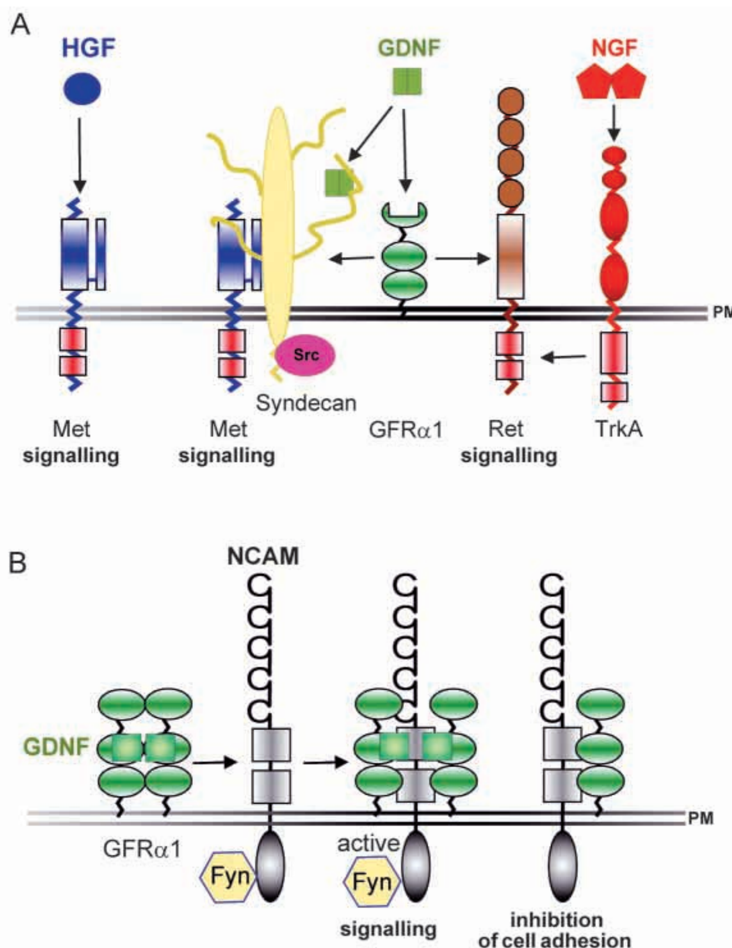

Fig. 2. Non-Ret signalling for GDNF and GFRα-independent signalling for RET. (A) GDNF promotes phosphorylation of Met. Its activation is indirect and is mediated by Src-type kinases. Various types of evidence suggest that GDNF is locally concentrated by heparan sulphate proteoglycans, such as syndecan. By contrast, RET is phosphorylated, by an unknown mechanism, through the activation of TrkA. Heparan sulphate proteoglycans may modulate GDNF signalling by local concentration of the ligand. (B) NCAM is an alternative signalling receptor for GFLs. It interacts with a GDNF-GFRα dimer leading to activation of Fyn, a Src-like kinase, for instance. It is not known whether Fyn activates Met in NCAM-mediated GDNF signalling.

significance of the GDNF-induced activation of Met is still unresolved.

NCAM is the second signalling receptor for GFLs

In many areas of the nervous system, and especially in the forebrain, cortex and inner ear, GFRα receptors are much more widely expressed than RET (Trupp et al., 1997; Kokaia et al., 1999; Ylikoski et al., 1999). This suggested that GFLs signal in neuronal and glial cells independently of RET in collaboration with other transmembrane protein(s). Carlos Ibañéz and co-workers noticed that the signalling pathways triggered by GDNF in an RN33B cell line expressing GFRα1, but not RET, significantly overlap with those triggered by NCAM. They have now demonstrated that NCAM functions as an alternative signalling receptor for GFLs (Paratcha et al., 2003). In the absence of GFRα proteins, GFLs interact with NCAM with low affinity. When GFRα1 is associated with NCAM, GDNF binds with high affinity to $p140^{NCAM}$ and activates in the cytoplasm the Src-like kinase Fyn and focal adhesion kinase FAK (Paratcha et al., 2003) (Fig. 2B). Interestingly, association of GFRα1 with NCAM also downregulates NCAM-mediated cell adhesion if GDNF is not present.

The ability of GFRα1 to modulate NCAM-mediated cell adhesion even in the absence of GDNF suggests independent roles for GFRα1-NCAM and GDNF-GFRα1-NCAM signaling. By binding to NCAM, GDNF stimulates Schwann cell migration and axonal growth in hippocampal and cortical neurons in a RET-independent fashion. These findings suggest that GFRα proteins and GFLs, interacting either together or alone with NCAM, use different signalling pathways to modulate both short- and long-range intercellular communication. Further studies are needed to reveal the in vivo roles of GDNF-NCAM and GDNF-GFRα-NCAM signalling, and to dissect the specific contribution and possible interplay of both RET and NCAM in signalling by GFLs. A recent study demonstrating that both in vitro and in vivo effects of GDNF on midbrain dopaminergic neurons are inhibited by an NCAM-blocking antibody further supports the physiological relevance of GDNF signalling through NCAM (Chao et al., 2003).

Ret signals independently of GFLs

Early studies clearly demonstrated that nerve growth factor (NGF) is the classical trophic factor acting on sympathetic superior cervical ganglion (SCG) neurons. During development, the survival of almost all SCG neurons depends on NGF and its receptor TrkA. During recent years, evidence indicating that GDNF, NRTN and ARTN also act on SCG neurons has accumulated (reviewed by Airaksinen and Saarma, 2002). Activation of the tyrosine kinase receptor TrkA by NGF is not needed for the survival of the postnatal sympathetic neurons, but it is required for their growth and for development of a mature neurotransmitter phenotype. The level of RET phosphorylation increases with postnatal age in sympathetic neurons in vitro and in vivo, although its function in these neurons during postnatal development has remained unclear. Surprisingly, NGF promotes the phosphorylation of the long isoform of RET independently of either GFLs or GFRα co-receptors (Fig. 2) (Tsui-Pierchala et al., 2002c). NGF promotes RET51 phosphorylation through a novel GFL-independent inter-receptor-tyrosine-kinase signalling mechanism, yielding enhanced growth, metabolism and gene expression. Since NGF activates only RET51 phosphorylation and does not activate the short isoform of RET, it is unlikely that NGF simply modulates the levels of GDNF protein. These surprising results show how growth factors and their receptors engage in crosstalk to form a network of inter-related trophic signals that guide development. The molecular mechanism of this crosstalk between Trk and RET is unknown, but it is apparently indirect.

GDNF regulates ureteric branching

Although originally identified as a neurotrophic factor, GDNF has turned out to be essential for kidney morphogenesis, where it is an inductive signal sent from the nephrogenic mesenchyme to the ureteric bud (reviewed by Cho and Dressler, 2003; Sariola et al., 2003). The inductive signalling during nephrogenesis is reciprocal. The ureteric bud induces epithelial differentiation of the nephrogenic mesenchyme, which in turn promotes branching of the bud. GDNF is expressed by the nephrogenic mesenchyme and RET is expressed by the adjacent tips of the branching ureteric bud (Hellmich et al., 1996; Suvanto et al., 1996). GFRα1 is expressed by both the nephrogenic mesenchyme and the ureteric bud (Sainio et al., 1997). In mice in which GDNF, RET or GFRα1 is knocked out, the kidneys show aplasia or severe hypodysplasia (Fig. 3) (reviewed by Sariola and Saarma, 1999). Metanephric development is initiated in 61% of RET-deficient embryos (Schuchardt et al., 1994), which suggests that other, non-Ret signalling systems are involved in ureteric branching. Indeed, ureteric branching of RET-deficient hypodysplastic kidney rudiments is partially restored by exogenous supplementation of GDNF (Popsueva et al., 2003). In accordance with this, the mice lacking GDNF show a more severe renal phenotype than RET- or GFRα1-deficient mice (Pichel et al., 1996).

Tissue culture studies have further implied that, although GDNF is essential for ureteric bud branching, other mesenchyme-derived signals are required. If microsurgically isolated ureteric buds are exposed to GDNF, they do not undergo branching. When such buds are recombined with heterologous mesenchyme, such as lung mesenchyme, the ureteric buds branch in response to GDNF (Sainio et al., 1997). The identity of this factor expressed by embryonic mesenchyme is unknown but, in cultures of isolated ureteric buds, heparin-binding growth-associated molecule (also known as pleiotrophin/osteoblast-stimulating factor 1) is required for GDNF-induced branching morphogenesis (Qiao et al., 1999).

Heparan sulphate proteoglycans may also be important for GDNF signalling in embryonic kidneys, because the effect of depriving kidneys of heparin sulphates is similar to that of knocking out GDNF or RET (Bullock et al., 1998). The transcription factors Pax2 and Eya1 control GDNF expression in differentiating nephrogenic mesenchymal cells (Xu et al., 1999; Brophy et al., 2001), whereas expression of RET by ureteric bud cells is indirectly regulated by retinoic acid (Batourina et al., 2001). NRTN, GFRα2 and NCAM are also expressed in the developing kidney, but they have no renal phenotype when knocked out (Cremer et al., 1994; Heuckeroth

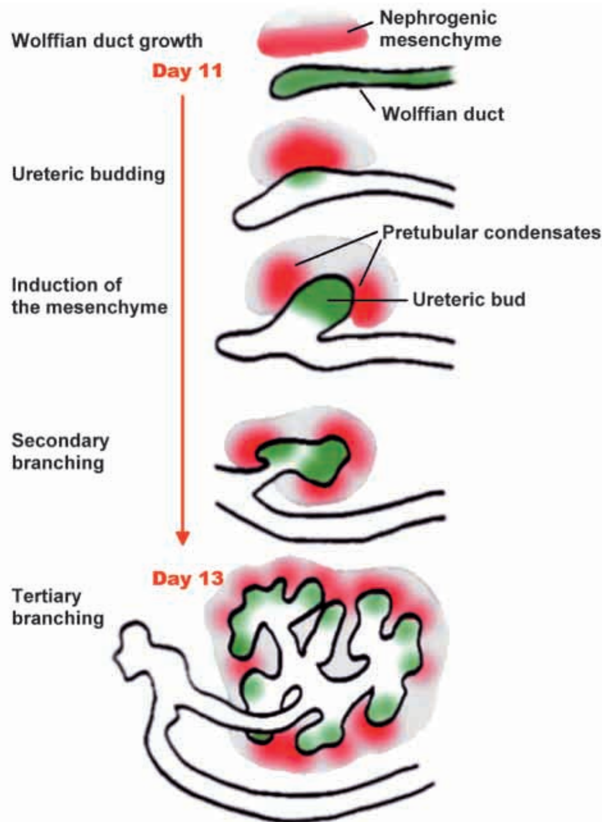

Fig. 3. GDNF regulates kidney development. GDNF (red) is expressed by the metanephric mesenchyme, and RET (green) by the Wolffian duct, from which the ureteric budding is promoted by GDNF. When the tip of the bud has induced two subsets of mesenchymal cells for epithelial differentiation, these cells strongly upregulate GDNF. This leads to formation of a double-gradient of GDNF around the ureteric tip and its branching.

et al., 1999; Rossi et al., 1999). Therefore, their in vivo roles in renal differentiation remain unclear.

… and spermatogenesis

GDNF is expressed in the testis by Sertoli cells that regulate spermatogenesis in a paracrine manner. RET and GFRα1 are displayed by a subset of undifferentiated spermatogonia, including the spermatogenic stem cells (Meng et al., 2000). Gene-targeted mice that have one *GDNF*-null allele show partial depletion of spermatogenic stem cells, whereas mice overexpressing GDNF show clusters of undifferentiated spermatogonia. Both GDNF and NRTN stimulate DNA synthesis in spermatogonia (Viglietto et al., 2000). In vivo data from transgenic mice show that GDNF, but not NRTN, contributes to the paracrine regulation of spermatogonial self-renewal and differentiation (Fig. 4) (Meng et al., 2000; Meng et al., 2001a). Reduced dosage of GDNF in $GDNF^{+/-}$ knock-out mice leads to excess differentiation of spermatogonia, their depletion and finally to Sertoli-cell-only histology. An increased dosage of GDNF allows undifferentiated spermatogonia to self-renew but not differentiate. Such GDNF-overexpressing mice are infertile and develop seminoma-like germ-line tumours in adulthood (Meng et al., 2001b). The regulation of GDNF expression by Sertoli cells is still poorly understood, but follicle-stimulating hormone is known to control the GDNF levels in Sertoli cells (Tadokoro et al., 2002). Also in the testis, a challenging issue will be to dissect the role of RET-dependent and -independent signalling.

RET receptor tyrosine kinase and GDNF in diseases

RET (rearranged during transformation) was originally identified following a transfection assay of NIH 3T3 fibroblasts with the DNA of a human T-cell lymphoma. It was soon discovered that mutations in the *RET* gene cause a number of different diseases. They are found in the majority of families who have multiple endocrine neoplasia type 2A (MEN2A) and type 2B (MEN2B) cancer syndrome. MEN2A is characterized by medullary thyroid carcinoma, pheochromocytoma (a malignancy of the adrenal medulla) and hyperparathyroidism. In MEN2B, half of the patients develop pheochromocytoma, but hyperparathyroidism is rare. Other features of MEN2B include marfanoid habitus, thickened corneal nerves, and neuromas of lips, tongue and gastrointestinal tract. In these sporadic or familial cancer syndromes, RET is constitutively activated by missense mutations, insertions or deletions either in the extracellular domain, critical for the receptor dimerization, or in the intracellular catalytic tyrosine kinase domains (reviewed by Hansford and Mulligan, 2000; Manié et al., 2001; Takahashi, 2001).

Various factors influence the type of RET mutation found in different cancers, such as exposure to radiation, age and the

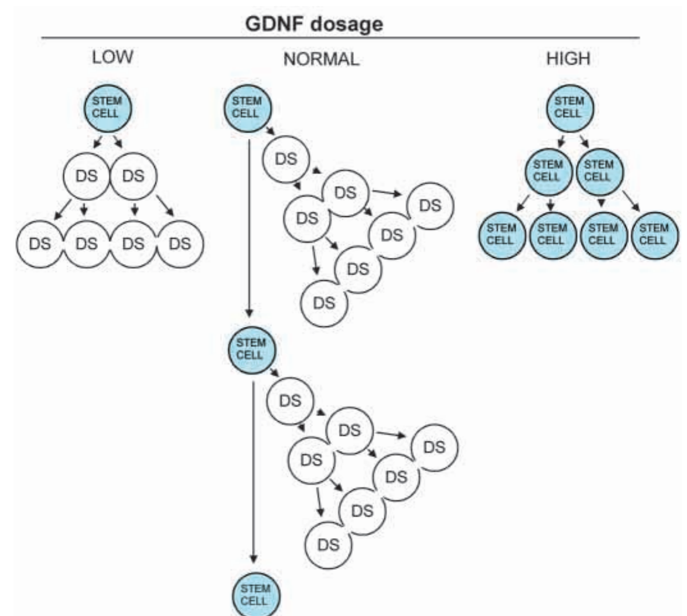

Fig. 4. Control of spermatogonial stem cell differentiation and self-renewal by GDNF dosage. GDNF is expressed by the Sertoli cells in the seminiferous tubules. These cells control sperm differentiation in a paracrine manner. When the GDNF level is low, the spermatogenic stem cells enter the differentiation pathway. When its level is high, the stem cells only self-renew and are unable to differentiate. In transgenic mice with loss- and gain-of-function of GDNF, both conditions lead to disturbed spermatogenesis.

histological type of the tumour. In medullary thyroid cancer, germline *RET* mutations are found in practically all familial cases, and somatic point mutations occur in nearly half of the sporadic cases. Papillary thyroid carcinomas (PTC) frequently show gene rearrangements, which give rise to chimeric genes referred to as *RET/PTC*. These rearrangements occur in almost half of papillary cancers (Hansford and Mulligan, 2000; Takahashi, 2001). Since both RET and Met are pathologically activated in various cancer forms, it is tempting to speculate about their possible synergistic effect in carcinogenesis.

Inactivating mutations in *RET* are common in Hirschsprung's disease, which is characterized by the absence of neuronal ganglia in various parts of the colon, leading to severe constipation and intestinal obstruction during childhood. Estimates of the frequency of *RET* mutations in Hirschsprung's patients vary, ranging in different populations from 5% to 50% (reviewed by Newgreen and Young, 2002). RET mutations are more common in the familial form than in sporadic Hirschsprung's disease. *GFRα* mutations have not been found in Hirschsprung's patients (Borrego et al., 2003), and they are unexpectedly not significant in the pathogenesis of the disease. Studies on the role of *GDNF* mutations in the pathogenesis of Hirschsprung's disease seem contradictory. Heterozygous $GDNF^{+/-}$ mice develop Hirschsprung-type intestinal obstruction (Shen et al., 2002), and four different mutations have been found in Hirschsprung's disease. At least two of them reduce the affinity of GDNF for GFRα1 (Eketjäll and Ibañéz, 2002). However, these GDNF mutations do not reduce the activation of RET (Borghini et al., 2002; Eketjäll and Ibañéz, 2002). NRTN has also been linked with the pathogenesis of Hirschsprung's disease. A mutation in *NRTN* has been found in a large pedigree with the disease. The mutation is not sufficient to cause Hirschsprung's disease but modifies the disease severity caused by a *RET* mutation in the pedigree (Doray et al., 1998).

Hirschsprung disease is a multigenic disease already associated with eight disease loci. The disease phenotype is modulated by genetic interactions between two or more disease genes, and there is low penetrance. Molecular genetic analyses have revealed that, in particular, the interaction between mutations in the genes encoding RET and endothelin receptor B (EdnrB) are central to the pathogenesis of Hirschsprung disease (Carrasquillo et al., 2002). In accordance with this, $RET^{+/-}$ heterozygous mice show no intestinal aganglionosis and *EdnrB*-null homozygotes show aganglionosis only in the very distal colon. When the $RET^{+/-}$ mice are crossed with mice carrying different combinations of *EdnrB*-null allele, the decreasing dosage of EdnrB dramatically increases the length of aganglionosis (MacCallion et al., 2003). Thus, genetic cross-talk between EdnrB and RET is essential for both normal development of the enteric nervous system and the pathogenesis of Hirschsprung disease.

Perspectives

GDNF is a potent neurotrophic factor that has restorative effects in a wide variety of rodent and primate models of Parkinson's disease (reviewed by Björklund et al., 2000). In early clinical studies no beneficial effects were observed, but side-effects were reported. Recently, GDNF was delivered directly into the putamen of five individuals with Parkinson's disease in a Phase I safety trial. This study demonstrated that direct intra-putamenal GDNF delivery in patients with Parkinson's disease can be tolerated for at least 1 year, leads to a significant increase in dopamine storage in the putamen, and improves the patient's clinical condition (Gill et al., 2003). Undoubtedly, it encourages further careful examination of the potential of GDNF as a treatment for Parkinson's disease.

GDNF is currently the most potent protein for the treatment of the Parkinson's disease. However, the molecular events leading to the degeneration of dopaminergic neurons are largely unclear. Importantly, death pathways activated in dopaminergic neurons by removal of neurotrophic factors, and GDNF in particular, have not been studied. Unravelling the intracellular cascades that are activated in GDNF-deprived dopaminergic neurons would offer a unique opportunity to develop pharmacological inhibitors that specifically block these death pathways but leave other pathways untouched. Use of low-molecular-weight drugs in therapy may be an interesting alternative to recombinant neurotrophic factors.

Other GFLs may also have therapeutic potential. Recent studies demonstrate that, in a rat model of Parkinson's disease, PSPN delivered to the brain by neural stem cells is as efficacious as GDNF at promoting both survival and neuritogenesis of midbrain dopamine neurons (Åkerud et al., 2002). Because the expression of PSPN receptor GFRα4 is much more restricted than that of the GDNF receptor GFRα1 (Lindahl et al., 2000), it is logical to assume that PSPN, even if used at higher concentration, would cause fewer side-effects than GDNF.

GFLs may also become useful for the treatment of drug addiction. It is well established that chronic administration of cocaine and morphine induce neurobiological changes in the ventral tegmental area (VTA) of the rat and mouse brain, the origin of the mesolimbic and mesocortical dopaminergic neurons. In animal studies, infusion of the VTA with GDNF blocks certain biochemical adaptations to chronic cocaine and morphine administration, as well as to the rewarding effects of cocaine. Most interestingly, chronic cocaine and morphine administration decreases RET-phosphorylation levels, suggesting that these drugs decrease signalling through endogenous GDNF pathways in the VTA (Messer et al., 2000). Although information about intracellular cascades triggered by GFLs is rapidly emerging, nothing is known about the neurotrophic-factor-induced signalling pathways involved in drug addiction. Since, in addition to GDNF, NRTN and PSPN protect dopaminergic neurons in the animal models of Parkinson disease, it will be of great interest to study whether NRTN and/or PSPN signalling can regulate adaptations to abused drugs.

The role of GDNF in the control of spermatogenesis is also intriguing, because GDNF is the first molecule that is known to control the cell fate decision of spermatogonial stem cells. GDNF signalling is therefore an attractive target for the development of male contraceptives. However, such efforts are severely shadowed by the carcinogenic consequences of deregulated RET activation.

The recently identified new GFL receptor NCAM, as well as crosstalk between GDNF-GFRα1-MET and NGF-TrkA-RET, make GDNF biology unexpectedly complicated. Horizontal interplay of unrelated receptor systems is being increasingly identified and undermining the classic view of the

ligand-receptor interaction as a one-way event. In spite of this complexity, GFLs offer a unique opportunity for development of drugs for treatment and possibly prevention of several diseases, particularly neurodegenerative diseases. However, GFL proteins are difficult and expensive to produce, they are often labile and their delivery to the target is complicated by the fact that they do not cross the blood-brain barrier. Therefore the next challenge is to find low-molecular-weight drugs that affect intracellular signalling pathways involving and mimicking the action of natural neurotrophic factors. The development of such drugs will be dependent on detailed understanding of the 3D structure of GFLs and their receptors, as well as the molecular, cellular and pathological aspects of their signalling mechanisms.

We thank Matthew Phillips for critical reading of the manuscript. Our original studies were supported by the Academy of Finland, Biocentrum Helsinki, HUCH research funding, TEKES, EU grant QLG3-CT-2002-01000 and the Sigrid Jusélius Foundation.

References

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

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

Åkerud, P., Holm, P. C., Castelo-Branco, G., Sousa, K., Rodriguez, F. J. and 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. Neusosci.* **21**, 205-222.

Arenas, E., Trupp, M., Åkerud, P. and Ibañéz, C. F. (1995). GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. *Neuron* **15**, 1465-1473.

Barnett, M. W., Fisher, C. E., Perona-Wright, G. and Davies, J. A. (2002). Signalling by glial cell line-derived neurotrophic factor (GDNF) requires heparan sulphate glycosaminoglycan. *J. Cell Sci*. **115**, 4495-4503.

Batourina, E., Gim, S., Bello, N., Shy, M., Clagett-Dame, M., Srinivas, S., Costantini, F. and Mendelsohn, C. (2001). Vitamin A controls epithelial/mesenchymal interactions through RET expression. *Nat. Genet.* **27**, 74-78.

Björklund, A., Kirik, D., Rosenblad, C., Georgievska, B., Lundberg, C. and Mandel, R. J. (2000). Towards a neuroprotective gene therapy for Parkinson 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.

Borghini, S., Bocciardi, R., Bonardi, G., Matera, I., Santamaria, G., Ravazzolo, R. and Ceccherini, I. (2002). Hirschsprung associated GDNF mutations do not prevent RET activation. *Eur. J. Human Genet.* **10**, 183-187.

Borrego, S., Fernández, R. M., Dziema, H., Niess, A., López-Alonso, M., Antinolo, G. and Eng, C. (2003). Investigation of germline GFRα4 mutations and evaluation of the involvement of GFRα1, GFRα2, GFRα3, and GFRα4 sequence variants in Hirschsprung disease. *J. Med. Genet.* **40**, e18.

Brophy, P. D., Ostrom, L., Lang, K. M. and Dressler, G. R. (2001). Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene. *Development* **128**, 4747-4756.

Bullock, S. L., Fletcher, J. M., Beddington, R. S. and Wilson, V. A. (1998). Renal agenesis in mice homozygous for a gene trap mutation in the gene encoding heparan sulfate 2-sulfotransferase. *Genes Dev*. **12**, 1894-1906.

Carrasquillo, M. M., McCallion, A. S., Puffenberger, E. G., Kashuk, C. S., Nouri, N. and 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.

Chao, C. C., Ma, Y. L., Chu, K. Y. and Lee, E. H. (2003). Integrin alphaV and NCAM mediate the effects of GDNF on DA neuron survival, outgrowth, DA turnover and motor activity in rats. *Neurobiol. Aging* **24**, 105-116.

Cho, E. A. and Dressler, G. R. (2003). Formation and development of nephrons. In *The Kidney: from Normal Development to Congenital Disease* (ed. P. Vize, A. S. Woolf and J. B. L. Bard), pp. 195-210. London: Academic Press.

Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S. et al. (1994). Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature* **367**, 455-459.

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

Doray, B., Salomon, R., Amiel, J., Pelet, A., Touraine, R., Billaud, M., Attié, T., Bachy, B., Munnich, A. and Lyonnet, S. (1998). Mutation of the RET ligand, neurturin, supports multigenic inheritance in Hirschsprung disease. *Hum. Mol. Genet.* **7**, 1449-1452.

Eketjäll, S. and Ibañéz, C. F. (2002). Functional characterisation of mutations in the GDNF gene of patients with Hirschsprung disease. *Hum. Mol. Genet.* **11**, 325-329.

Fisher, C. E., Michael, L., Barnett, M. W. and Davies, J. A. (2001). Erk MAP kinase regulates branching morphogenesis in the developing mouse kidney. *Development* **128**, 4329-4338.

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

Gill, S. S., Patel, N. K., Hotton, G. R., O'Sullivan, K., McCarter, R., Bunnage, M., Brooks, D. J., Svendesen, C. N. and Heywood, P. (2003). Direct brain infusion of glial cell line-derived neurotrophic factor in Parkisnson disease. *Nat. Med.* **9**, 589-595.

Grondin, R. and Gash, D. M. (1998). Glial cell line-derived neurotrophic factor (GDNF): a drug candidate for the treatment of Parkinson's disease. *J. Neurol.* **245**, 35-42.

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

Hellmich, H. L., Kos, L., Cho, E. S., Mahon, K. A. and Zimmer, A. (1996). Embryonic expression of glial cell-line derived neurotrophic factor (GDNF) suggests multiple developmental roles in neural differentiation and epithelial-mesenchymal interactions. *Mech. Dev.* **54**, 95-105.

Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., Armanini, M., Simmons, L., Moffet, B., Vandlen, R. A. and Rosenthal, A. (1994). GDNF: A potent survival factor for motoneurons present in peripheral nerve and muscle. *Science*, **266**, 1062-1064.

Heuckeroth, R. O., Enomoto, H., Grider, J. R., Golden, J. P., Hanke, J. A., Jackman, A., Molliver, D. C., Bardgett, M. E., Snider, W. D., Johnson, E. M. and 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.

Kaplan, D. R. and Miller, F. D. (2000). Neurtrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* **10**, 381-391.

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

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

Lin, L. F., Doherty, D. H., Lile, J. D., Bektesh, S. and Collins, F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurones. *Science* **260**, 1130-1132.

Lindahl, M., Timmusk, T., Rossi, J., Saarma, M. and Airaksinen, M. S. (2000). Expression and alternative splicing of mouse *Gfrα4* suggests roles in endocrine cell development. *Mol. Cell. Neurosci.* **15**, 522-533.

MacCallion, A. S., Stames, E., Conlon, R. A. and Chakravarti, A. (2003). Phenotype variation in two-locus mouse models of Hirschsprung disease: Tissue-specific interaction between Ret and Ednrb. *Proc. Natl. Acad. Sci. USA* **100**, 1826-1831.

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

Meng, X., Lindahl, M., Hyvönen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J. G., Westphal, H., Saarma, M. and Sariola, H. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* **287**, 1489-1493.

Meng, X., Pata, I., Pedrono, E., Popsueva, A., de Rooij, D. G., Jänne, M.,

Rauvala, H. and Sariola, H. (2001a). Transient disruption of spermatogenesis by deregulated expression of neurturin in testis. *Mol. Cell. Endocrinol.* **183**, 33-39.

Meng, X., de Rooij, D. G., Westerdahl, K., Saarma, M. and Sariola, H. (2001b). Promotion of seminomatous tumors by targeted overexpression of glial cell line-derived neurotrophic factor in mouse testis. *Cancer Res.* **61**, 3267-3271.

Messer, J. C., Eisch, A. J., Carlezon, W. A., Jr, Whisler, K., Shen, L., Wolf, D. H., Westphal, H., Collins, F., Russell, D. S. and Nestler, E. J. (2000). Role of GDNF in biochemical and behavioral adaptations to drug abuse. *Neuron* **26**, 247-257.

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

Paratcha, G. and Ibañéz, C. F. (2002). Lipid rafts and the control of neurotrophic factor signaling in the nervous system: variations on a theme. *Curr. Opin. Neurobiol.* **12**, 542-549.

Paratcha, G., Ledda, F. and Ibañéz, C. F. (2003). The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. *Cell* **113**, 867-879.

Peterziel, H., Unsicker, K. and Krieglstein, K. (2002). TGFβ induces GDNF responsiveness in neurons by recruitment of GFRα1 to the plasma membrane. *J. Cell Biol*. **159**, 157-167.

Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J., Sariola, H. and Westphal, H. (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73-76.

Popsueva. A., Poteryaev, D., Arighi, E., Meng, X., Angers-Loustau, A., Kaplan, D., Saarma, M. and Sariola, H. (2003). GDNF promotes tubulogenesis of GFR1-expressing MDCK cells by Src-mediated phosphorylation of Met receptor tyrosine kinase. *J. Cell Biol.* **161**, 119-129.

Poteryaev, D., Titievsky, A., Sun, Y. F., Thomas-Crusells, J., Lindahl, M., Billaud, M., Arumäe, U. and Saarma, M. (1999). GDNF triggers a novel ret-independent Src kinase family-coupled signaling via a GPI-linked GDNF receptor alpha1. *FEBS Lett.* **463**, 63-66.

Qiao, J., Sakurai, H. and Nigam, S. K. (1999). Branching morphogenesis independent of mesenchymal-epithelial contact in the developing kidney. *Proc. Natl. Acad. Sci. USA* **96**, 7330-7335.

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

Saarma, M. and Sariola, H. (1999). Other neurotrophic factors: glial cell line-derived neurotrophic factor (GDNF). *Microscop. Res. Tech.* **45**, 292-302.

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

Sariola, H. and Saarma, M. (1999). GDNF and its receptor in the regulation of ureteric branching. *Int. J. Dev. Biol.* **43**, 413-418.

Sariola, H., Sainio, K. and Bard, J. (2003). Fates of the metanephric mesenchyme. In *The Kidney: from Normal Development to Congenital Disease* (ed. P. Vize, A. S. Woolf and J. B. L. Bard), pp. 181-194. London: Academic Press.

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

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

Suvanto, P., Hiltunen, J. O., Arumäe, U., Moshnyakov, M., Sariola, H., Sainio, K. and 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.

Tadokoro, Y., Yomogida, K., Ohta, H., Tohda, A. and Nishimune, Y. (2002). Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. *Mech. Dev.* **113**, 29-39.

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

Tanaka, M., Xiao, H. and Kiuchi, K. (2002). Heparin facilitates glial cell line-derived neurotrophic factor signal transduction. *NeuroReport* **13**, 1913-1916.

Trupp, M., Belluardo, N., Funakoshi, H. and Ibañéz, C. F. (1997). Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), cret proto-oncogene, and gdnf receptor-alpha indicates multiple mechanisms of trophic actons in the adult rat CNS. *J. Neurosci.* **17**, 3554-3567.

Trupp, M., Scott, R., Whittemore, S. R. and Ibañéz, C. F. (1999). RET-dependent and -independent mechanisms of glial cell line-derived neurotrophic factor signaling in neuronal cells. *J. Biol. Chem.* **274**, 20885-20894.

Tsui-Pierchala, B. A., Encinas, M., Milbrandt, J. and Johnson, E. M., Jr (2002a). Lipid rafts in neuronal signaling and function. *Trends Neurosci.* **25**, 412-417.

Tsui-Pierchala, B. A., Ahrens, R. C., Crowder, R. J., Milbrandt, J. and Johnson, E. M., Jr (2002b). The long and short isoforms of RET function as independent signaling complexes. *J. Biol. Chem.* **277**, 34618-34625.

Tsui-Pierchala, B. A., Milbrandt, J. and Johnson, E. M. (2002c). 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.

Viglietto, G., Dolci, S., Bruni, P., Baldassarre, G., Chiariotti, L., Melillo, R. M., Salvatore, G., Chiappetta, G., Sferratore, F., Fusco, A. and Santoro, M. (2000). Glial cell line-derived neutrotrophic factor and neurturin can act as paracrine growth factors stimulating DNA synthesis of RET-expressing spermatogonia. *Int J. Oncol.* **16**, 689-694.

Xu, P. X., Adams, J., Peters, H., Brown, M. C., Heaney, S. and Maas, R. (1999). Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat. Genet.* **23**, 113-117.

Ylikoski, J., Pirvola, K., Virkkala, J., Suvanto, P., Liang, X.-Q., Magal, E., Altschuler, R., Miller, J. M. and Saarma, M. (1998). Guinea pig auditory neurons are protected by glial cell line-derived growth factor from degeneration after noise trauma. *Hearing Res*. **124**, 17-26.