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GDNF AND p75 NEUROTROPHIN RECEPTOR IN DEVELOPMENT AND DISEASE

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Academic Dissertation

To be publicly discussed with the permission of the Medical Faculty of the University of Helsinki, in the Niilo Hallman auditorium of the Hospital of Children and Adolescents, Stenbäckinkatu 11, Helsinki, on July 3rd, at 12 noon

Helsingin yliopiston verkkojulkaisut
Helsinki 1998
ISBN 951-45-8661-1 (PDF version)

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Ancient coins from the predoctoral era to honor the

MUSAE FENNIAE

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
BDNF	brain-derived neurotrophic factor
bp	base pair
CFLP	cleavase fragment length polymorphism
cM	centimorgan
cDNA	complementary DNA
CNS	central nervous system
CNTF	ciliary neurotrophic factor
DGGE	denaturing gel gradient electrophoresis
DNA	deoxyribonucleic acid
E	embryonic day
EGF	epidermal growth factor
EST	expressed sequence tag
FGF	fibroblast growth factor
FISH	fluorescent *in situ* hybridization
GDNF	glial cell line -derived neurotrophic factor
GFRa	GDNF family receptor alpha
HA	heteroduplex analysis
kb	kilobase pairs
kD	kilodalton
K_d	dissociation constant
LIF	leukemia inhibitory factor
MAPK	mitogen activated protein kinase
MEN2	multiple endocrine neoplasia type 2
MPTP	1-methyl-4phenyl-1,2,5,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NT	neurotrophin
NTN	neurturin
p75NTR	p75 neurotrophin receptor
PCR	polymerase chain reaction
PSP	persephin
RFLP	restriction fragment length polymorphism
RH	radiation hybrid
RNA	ribonucleic acid
RT	reverse transcription
SSC	sodium chloride-sodium citrate
SSCP	single strand conformation polymorphism
TGFb	transforming growth factor beta

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals, and some unpublished results:

I Durbec P, Marcos-Gutierrez C, Kilkenny C, Grigoriou M, Wartiovaara K, Suvanto P, Smith D, Ponder B, Costantini F, Saarma M, Sariola H and Pachnis V (1996): GDNF signalling through the Ret receptor tyrosine kinase. *Nature* 381:789-793.

II Suvanto P, Wartiovaara K, M Lindahl M, Arumäe U, Moshnyakov M, Horelli-Kuitunen N, Airaksinen MS, Palotie A, Sariola H and Saarma M (1997): Cloning, mRNA distribution and chromosomal localisation of the gene for glial cell line-derived neurotrophic factor receptor -b, a homologue to GDNFR-a. *Human Molecular Genetics* 6:1267-1273.

III Wartiovaara K, Salo M, Sainio K, Rintala R and Sariola H (1998): Distribution of Glial cell line-derived neurotrophic factor mRNA in human colon suggests roles for muscularis mucosae in innervation. Submitted to Journal of Pediatric Surgery.

IV 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 ureteric epithelium. *Development* 124: 4077-4087.

V Wartiovaara K, Paavola P, Suvanto P, Paulin L, Saarma M, Palotie L, and Sariola H (1997): Exclusion of the low-affinity nerve growth factor receptor p75 gene as a candidate gene for Meckel syndrome. *Clinical Dysmorphology* 6: (3) 213-217.

VI Wartiovaara K, Hytönen M, Vuori M, Rinne J, and Sariola H (1998): Mutation analysis of the Glial cell line -derived neurotrophic factor gene in Parkinson's disease. *Experimental Neurology*, in press.

SUMMARY

Neurotrophic factors are secreted molecules that are responsible for many functions in the development, differentiation and survival of mainly neural but also other tissues. The glial cell line-derived neurotrophic factor (GDNF), a potent survival factor for the midbrain dopaminergic neurons, was found to signal through a tyrosine kinase receptor, Ret. This receptor complex includes other, glycosylphosphoinositol-linked proteins, termed GDNF family receptor alphas (GFRαs), that form a family of homologous molecules involved in the signaling of GDNF and its relatives. GFRα-2 was characterized in this study.

GDNF and Ret are crucial for the development of the kidney and the enteric nervous system of the digestive tract. GDNF mRNAs were found in the muscularis mucosae of the developing human colon whereas the cognate receptors were expressed in the ganglia of the entire digestive tract. In the kidney, GDNF was found to be responsible for the budding of the ureter from the Wolffian duct.

The gene encoding GDNF was analyzed for mutations in diseases that are closely linked to the biology of GDNF or its receptors. No disease-associated mutations were found in the samples from the patients suffering from Hirschsprung's disease (aganglionic megacolon), renal aplasia or Parkinson's disease.

Another important group of neurotrophic factors are called neurotrophins. They all bind to p75 neurotrophin receptor (p75NTR) and to the different members of the receptor tyrosine kinases. p75NTR is expressed in the kidney and nervous system, as well as in non-neuronal cells in some other tissues. The p75NTR gene was analyzed in the patients of a lethal disorder, Meckel syndrome, with a developmental defect in the renal, limb and brain morphogenesis. One heterozygous polymorphism was found and shown not to be the disease mutation. The p75NTR gene was then mapped outside the Meckel locus.

REVIEW OF THE LITERATURE

NEUROTROPHIC FACTORS

Introduction

In embryogenesis, the cells send and receive signals to develop towards a more differentiated stage, to divide or to die. The successful ways to create different functional units, like the neuronal system, have been conserved in evolution and very similar mechanisms are responsible for the development and survival of neurons in most species from insects to man. These neuronal differentiation and survival molecules include the neurotrophic molecules, some of which are also active in non-neuronal embryonic tissues, or re-activated in adulthood in tissue renewal or regeneration. In human disease, the neurotrophic factors and the genes encoding for them have been studied in various developmental disorders, birth defects and neurodegenerative diseases.

Like most signaling molecules, the neurotrophic factors can be divided into families that are formed of closely related molecules (Table 1) (Korsching 1993; Arumäe et al., 1997). A prototypic neurotrophic factor, such as nerve growth factor (NGF), is a secreted, target-derived molecule that binds to a transmembrane receptor (Barbacid 1995; Segal and Greenberg, 1996) on the cell surface. The receptor then dimerazes and is activated by transphosphorylation of the catalytic intracellular domain, which starts a complex intracellular signaling cascade leading to immediate, early and late transcriptional changes in the target cell.

Some of the neurotrophic factors are secreted, but not derived from a distant target tissue. These molecules, such as the ciliary neurotrophic factor (CNTF) have an auto- or paracrine effect on neuronal cells. CNTF is structurally related to cytokines, such as leukemia inhibitory factor, oncostatin M, interleukin –6, interleukin –11 and cardiotrophin (Sariola et al., 1994). Also, some molecules that are not secreted and have initially been studied as adhesion molecules have later been found to mediate neuronal cell differentiation, for instance. The integrins and Eph family of tyrosine kinases are two types of receptors that are activated by membrane bound ligands like ELF-1 and repulsive axon guidance signal (Segal and Greenberg, 1996).

Table 1. Families of neurotrophic factors

Neurotrophins
- Nerve Growth Factor (NGF)
- Brain-Derived Neurotrophic Factor (BDNF)
- Neurotrophin 3 (NT3)
- Neurotrophin 4/5 (NT4/5)

Neuropoietins
- Ciliary Neurotrophic Factor (CNTF)
- Leukemia Inhibitory Factor (LIF)

Insulin-like Growth Factors 1-2 (IGF-1, IGF-2)

Transforming Growth Factors
- Transforming Growth Factor α (TGFα)
- Transforming Growth Factor β 1-3 (TGFβ1, TGFβ2, TGFβ3)
- Glial Cell Line-Derived Neurotrophic Factor (GDNF)
- Neurturin (NTN)
- Persephin (PSP)

Fibroblast Growth Factors
- Acidic Fibroblast Growth Factor (FGF-1)
- Basic Fibroblast Growth Factor (FGF-2)
- Fibroblast Growth Factor-5 (FGF-5)

Other factors
- Platelet-Derived Growth Factor (PDGF)
- Stem Cell Factor (SCF)

The NGF family

Neurotrophins

Neurotrophins were originally characterized because of their functions in differentiation and survival of neurons, although many of them are now known to affect other cell types as well (Levi-Montalcini 1987; Sariola et al., 1994; Barbacid 1995; Segal and Greenberg, 1996). The first neurotrophin, NGF, was isolated from snake venom as early as 1956 based on its ability to promote the neurite growth of sympathetic neurons in chicken (Cohen and Levi-Montalcini, 1956). NGF prevented the neuronal death caused by 6-hydroxydopamine (6-OHDA) (Levi-Montalcini et al., 1975), vinblastine or surgical transectomy (Hendry 1975) of the postganglionic axons of the superior cervical ganglion. The ability of neurons to grow axons towards increasing concentrations of NGF (Gundersen and Barrett, 1979; Gundersen and Barrett, 1980) finally led to the target field hypothesis of the development of innervation. This theory (Korsching 1993; Lewin and Barde, 1996) postulates that, as most of the developing neurons die during embryogenesis, their survival is dependent on the ability of the neuron to compete for the limited amount of a trophic factor. Neurons that fail to grow axons to the target, die apoptotically, as shown in Figure 1.

After the discovery of NGF by Levi-Montalcini and co-workers in the 50s, several other members of the NGF family have been found. The group of neurotrophins comprises now of the brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin -3 (NT3) (Ernfors et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990) and neurotrophin -4/5 (NT4/5) (Berkemeier et al., 1991; Hallbook et al., 1991). Neurotrophin -6 (NT6) has been found in teleost fish (Götz et al., 1994).

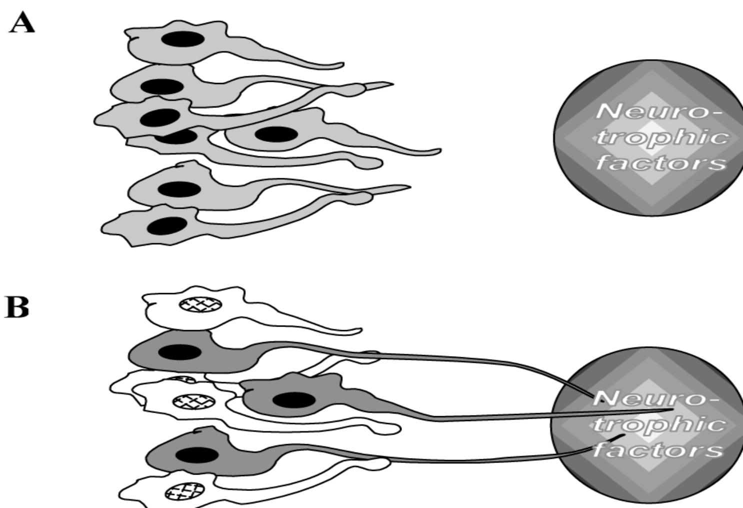


Figure 1. The target field theory.
A.The axons of neurons grow towards the higher concentration of the trophic factor, secreted by the target tissue (shaded ellipse).
B. Neurons that succeed to get in contact with the target tissue (gray) are maintained, but others (white) die.

All neurotrophins (Sariola et al., 1994; Barbacid 1995; Lewin and Barde, 1996; Segal and Greenberg, 1996) have similar biochemical characteristics. They are synthesized as precursor proteins, then modified to mature forms, secreted, and they all act as dimers. Approximately half of the amino acid residues in the mature protein are common to all neurotrophins and they all share six cysteine residues at identical positions. This leads to the formation of the disulfide bridges and the similar tertiary structure of the molecules. The neurotrophins are able to form stable heterodimers in vitro (Jungbluth et al., 1994), although the role of hetero-dimerization in vivo is not known.

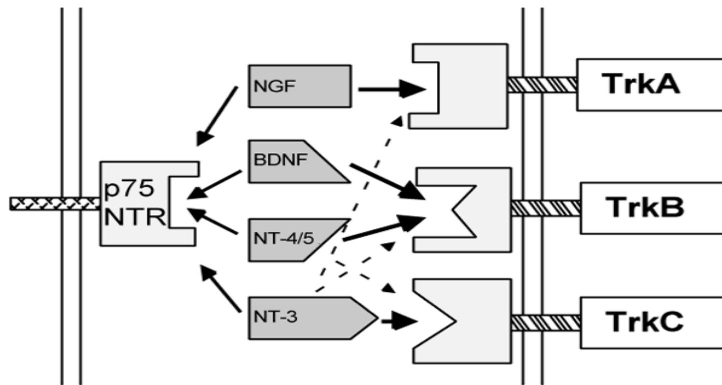


Figure 2. The neurotrophin receptors. Preferred receptors are shown with bold arrows, the dashed arrows show weak interactions. All neurotrophins bind to p75NTR.

Neurotrophin receptors

The neurotrophins bind to two types of receptors, tyrosine kinase receptors TrkA, TrkB or TrkC and a common neurotrophin receptor p75 (p75NTR) that has no tyrosine kinase domain (Chao and Hempstead, 1995; Greene and Kaplan, 1995; Segal and Greenberg, 1996). NGF binds specifically to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC. In addition to these specific receptors, all neurotrophins bind to the p75NTR (Figure 2).

Trk receptors have different splice variants, some of which lack the catalytic tyrosine kinase intracellular domain but the function of these non-catalytic variants is not known (Barbacid 1995). The signal transduction of Trk receptors has been intensively studied and many downstream target molecules identified (Kaplan and Miller, 1997).

The p75NTR belongs to the tumor necrosis factor receptor family and was the first identified neurotrophin receptor (Johnson et al, 1986). Although it does not have a catalytic intracellular tyrosine kinase domain, it is capable of mediating the neurotrophin signals. This happens both by its modulating TrkA signaling and independently of Trk:s (Kaplan and Miller, 1997). The ligand binding

ofp75NTR increases the high-affinity TrkA binding sites, enhances TrkA autophosphorylation and selectivity for neurotrophin ligands. The Trk-independent pathway of p75NTR increases intracellular ceramide levels and further activates NFkB transcription factor (Carter et al., 1996) and JNK kinase (Casaccia-Bonnefil et al., 1996). Conversely, TrkA activation inhibits p75NTR-mediated signaling, but the mechanism of this inhibition is unclear (Kaplan and Miller, 1997).

Neurotrophins and their receptors in development and disease

All neurotrophins stimulate neurite outgrowth and survival of certain neurons or neural crest cells. The expression pattern of neurotrophins are only partially overlapping, they often affect different subsets of neurons and show some specific biological functions (Lewin and Barde, 1996). The roles of neurotrophins and their receptors in development have been approached by targeted gene disruption (Table 2).

Despite severe phenotypes in the peripheral nervous system and the high ligand-receptor phenotype correlation (with some exceptions), the knockout mice have not solved the question of the roles of neurotrophins in the central nervous system (CNS) differentiation (Snider 1994; Barbacid 1995). As most of these mutant mice die during the first weeks of life, while the CNS is still developing, the possible consequences of the disrupted genes cannot be studied by this approach. It may be that the development of the CNS has been secured with a back-up system that, for example, allows the different neurotrophins to compensate for the loss of each other. We do not know all the functions of neurotrophins and a recent surprising finding is that in contrast to the well documented survival effects of neurotrophins, NGF can induce apoptosis in some neuronal cells (Muragaki et al., 1997).

Table 2. Main defects observed in the targeted disruptions of neurotrophins and their receptors (Snider 1994; Barbacid 1995)

Phenotype	p75	NGF	TrkA	BDNF	TrkB	NT-3	TrkC
Neuronal loss	some	severe	severe	severe	severe	marked	marked
Sup.cerv.ganglia	no	95%	95%	no	no	50%	25%
Trigeminal ganglia	no	70%	70%	40%	90%	60%	?
Nodose-petrosal g.	?	no	no	85%	?	40%	?
Vestibular ganglia	?	?	?	30%	30%	20%	?
Dorsal root ganglia	yes	70%	70%	no	no	65%	20%
Function							
Nociception	partial	low	low	normal	normal	normal	normal
Balance	normal	normal	normal	poor	?	normal	normal
Proprioception	normal	normal	normal	normal	normal	poor	poor

Neurotrophin receptors have been linked to human diseases. The TrkA gene was originally found as an oncogene in colon cancer (Martin-Zanca et al., 1986) and its translocations are common in papillary thyroid carcinoma (Bongarzone et al., 1989).
Recently, a mutation in the TrkA gene was found to cause congenital insensitivity to pain with anhidrosis (CIPA) syndrome (Indo et al., 1996) that closely resembles the phenotype of the TrkA -deficient mice. The TrkA and TrkC expression also have prognostic significance for favorable outcome in neuroblastoma and medulloblastoma, respectively (Nakagawara et al., 1993; Segal et al., 1994). No disease associations have been described either for the TrkB gene, or the genes for p75NTR or any of the neurotrophins. The chromosomal localizations of the neurotrophin and their receptor genes are shown in Table 3.

Table 3. Chromosomal localizations of neurotrophin and their receptor genes

Gene	Human locus	Reference
NGF	1p22	(Francke et al., 1983)
BDNF	11p13	(Maisonpierre et al., 1991)
NT-3	12p13	(Maisonpierre et al., 1991)
NT-4/5	19q13.3	(Berkemeier et al., 1992)
p75NTR	17q21-q22	(Huebner et al., 1986)
TrkA	1q21-q22	(Weier et al., 1995)
TrkB	9q22.1	(Nakagawara et al., 1995)
TrkC	15q25	(Valent et al., 1997)

GDNF family of neurotrophic factors

GDNF was found in a classical neuronal survival approach used in the search for secreted neurotrophic factors (Lin et al., 1993). The new, potent neurotrophic molecule was identified because of the ability of the conditioned media of glial cell line cultures to promote the survival of dopaminergic neurons. Purification and cloning of GDNF revealed it to be a member of the transforming growth factor beta (TGFb) superfamily. Recently, two new members of the GDNF family, neurturin (NTN) and persephin (PSP) have been cloned and characterized (Kotzbauer et al., 1996; Milbrandt et al., 1998).

Like neurotrophins, also GDNF, NTN and PSP are first synthesized as precursor polypeptides and then processed to mature proteins and secreted (Rosenthal 1997). They function as disulfide-bonded homodimers and share the conserved cysteine residues of the TGFb superfamily although the overall amino acid homology to TGFb-1, for example, is not more than 20% (Milbrandt et al., 1998). GDNF, NTN and PSP are structurally close to each other and their identity at the amino acid level is about 40%. They share the sequence suggesting a common ”cysteine knot” structure formed by three disulfide bonds and the potential receptor binding surfaces have been identified based on the X-ray crystal structure of GDNF (Eigenbrot and Gerber, 1997).

In this study we found that the functional receptor for GDNF is the Ret receptor ty-

rosine kinase (I). NTN was later also shown to signal through Ret (Buj-Bello et al. 1997, Klein et al. 1997). The receptor complex binding GDNF and NTN is composed of Ret and a glycosyl-phosphoinositol-linked GDNF family receptor alpha (GFRα) protein (Rosenthal 1997). The downstream pathways are most likely common for all GDNF family factors since at least GDNF or NTN binding to Ret both activate the MAP kinase pathway. The chromosomal loci of the GDNF family members and their receptor genes are listed in Table 4.

Table 4. Chromosomal loci of the GDNF family member and receptor genes

Gene	Human locus	Reference
GDNF	5p12-p13.1	(Bermingham et al., 1995; Schindelhauer et al., 1995)
NTN	19p13.3	(Heuckeroth et al., 1997)
PSP	unknown	
Ret	10q11.2	(Ishizaka et al., 1989)
GFRα-1	10q26	(Gorodinsky et al., 1997)
GFRα-2	8p21-p22	(II)
GFRα-3	5q31.1-31.3	(Masure et al., 1998)

The biological properties and expression patterns of the GDNF family neurotrophic factors differ. They all have a neurotrophic effect on dopaminergic and motoneurons, but GDNF and NTN also seem to support peripheral neuronal types. At the moment, the functions of GDNF are best characterized and will be discussed further.

GDNF gene

The coding region of the GDNF gene is 633 bp encoding for a 211 amino acid precursor polypeptide from which the mature GDNF of 134 amino acids is processed by proteolytic cleavage (Lin et al., 1993). Another form of alternatively spliced GDNF mRNA lacking 78 bp in the prepro-region has also been found (Suter-Crazzolara and Unsicker, 1994), but since both isoforms produce the same mature GDNF, the function of the different splicing is unknown. The human gene has been mapped to the chromosome 5p12- p13.1 (Schindelhauer et al., 1995) and it has two coding exons that are more than 10 kb apart. The full-length GDNF mRNA has both 5’ and 3’ untranslated regions that make it up to 4.4 kb long (unpublished data).

GDNF in the nervous system

GDNF mRNA expression has been analyzed in many tissues and species by *in situ* hybridization (Hellmich et al., 1996; Suvanto et al., 1996), RNase protection (Trupp et al., 1995), reverse transcription polymerase chain reaction (RT-PCR) (Choi-Lundberg and Bohn, 1995) and heterozygous transgenic GDNF- *lacZ* mice (Sanchez et al., 1996). GDNF is expressed in many areas of the CNS during development and adulthood. The first expression site is in the rostral part of the mouse neural plate at the embryonic day (E)7.5 and then continues in the anterior neuroectoderm until E10.5 (Hellmich et al., 1996; Suvanto et al., 1996). Thereafter, the expression becomes more restricted to the lateral domains of rostral and caudal hindbrain. Only at late embryonic and newborn stages, as well as in the adult, is the mRNA expression seen in the striatum and other areas of developing dopaminergic system (Choi-Lundberg and Bohn, 1995; Trupp et al., 1997).

GDNF specifically affects cultured dopaminergic midbrain neurons without affecting other neurons or glia of substantia nigra (Lin 1996). It increases dopaminergic cell number, dopamine uptake, cell size and neurite length without any change in γ-aminobutyrate acid (GABA) or serotonin uptake or overall number of neurons.

Importantly for the potential clinical use, GDNF also protects these neurons from various toxic agents as will be discussed later with the animal models for Parkinson's disease.

GDNF affects motor neurons by both *in vivo* and *in vitro*. In cell culture, 0.1 pg/ml of GDNF increases motoneuron survival and cell number, neurite outgrowth and choline acethyltransferase (ChAT) activity (Henderson et al., 1994). The motoneurons can also be rescued from axotomy-induced or natural cell death by GDNF in mouse, rat and avian models (Henderson et al., 1994; Oppenheim et al., 1995). This response is not limited to developing motor neurons, but is also seen in the neurons of adult animals (Li et al., 1995; Yan et al., 1995) supporting the idea of the applicability of GDNF as a potential therapeutic agent in motor neuron diseases, such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy or in ischemic and mechanic injuries of the spinal cord.

GDNF in the kidney

GDNF mRNA is expressed in the mesonephric kidney that later regresses (Hellmich et al., 1996; Suvanto et al., 1996). The development of the metanephric, permanent mammalian kidney, is initiated by a signal from the nephrogenic mesenchyme that induces the Wolffian duct to produce the ureteric bud. The ureter then invades the mesenchyme and divides, and its tips induce the condensation and epithelialization of the adjacent mesenchyme into excretory tubules (Saxén, 1987). At that time, GDNF mRNA is abundantly expressed in the condensing mesenchymal cells around the invading and branching ureteric bud (Choi-Lundberg and Bohn, 1995; Hellmich et al., 1996; Suvanto et al., 1996). The expression follows the "nephrogenic zone" of condensing subcapsular cells and is downregulated in the differentiated cells that undergo epithelialization. GDNF mRNA is expressed also in the precursors of Sertoli cells in the testis, but not in the ovaries (Hellmich et al., 1996; Suvanto et al., 1996).

Already the strictly specific expression pattern of GDNF during nephrogenesis suggested a crucial role for it in urogenital development (Choi-Lundberg and Bohn, 1995; Hellmich et al., 1996; Suvanto et al., 1996). This was later verified through knockout mice experiments (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). The mode of action of GDNF in the ureter budding is shown in more detail in this study.

GDNF in other organs

The developing gastrointestinal tract expresses GDNF mRNA from the esophagus to the rectum (Hellmich et al., 1996). At first, the expression is seen in the undifferentiated mesenchymal layer that later on becomes the smooth muscle of the gut that continuously expresses GDNF transcripts. During development, the expression of GDNF is also seen in a variety of other mesenchymal tissues such as limb buds, cartilage and derivatives of the pharyngeal pouches, although its function in these sites is not understood (Hellmich et al., 1996; Suvanto et al., 1996).

Transgenic GDNF –mice

Four different transgenic mice strains have been constructed to study GDNF function (Moore et al., 1996; Pichel et al., 1996;

Sanchez et al., 1996; Nguyen et al., 1998). Two different GDNF -deficient mouse strains show reduced number of motoneurons by 20% to 30% in the trigeminal ganglia and spinal cord (Moore et al., 1996; Sanchez et al., 1996). This supports the hypothesis of GDNF functions as a muscle-derived survival factor for motoneurons that are dependent also on other factors, like cardiotrophin (deLapeyriere and Henderson, 1997; Arce et al., 1998). This effect is verified by the transgenic mouse line overexpressing GDNF under the muscle-specific myogenin promotor (Nguyen et al., 1998). The neuromuscular junctions of these mice are hyperinnervated for several weeks after birth in GDNF expression-dependent manner, suggesting that the normally occurring cell death is delayed.

The development of the dopaminergic neurons in GDNF null mice is not abnormal (Moore et al., 1996; Sanchez et al., 1996), possibly, because the homozygous mice die on the first postnatal day before the maturation of the dopaminergic system. No defects have been observed in the number of tyrosine hydroxylase-positive dopaminergic neurons in the A9-A10 brain regions where dopaminergic neurons develop or in the floorplate, the striatum or the basal ganglia. Also, the neurons within the locus coeruleus, a noradrenergic nucleus severely impaired in neurodegenerative diseases, show no loss in cell number although they have been shown to be GDNF-responsive in chemical-induced degeneration rescue studies (Arenas et al., 1995).

Consistent with the observation of GDNF promoting the survival of chick embryonic sensory neurons *in vitro*, the petrosal-nodose, sympathetic superior cervical and dorsal root ganglia of GDNF -deficient mice show reduction in both cell number and size (Moore et al., 1996). However, not all autonomic or sensory ganglia are affected and, for instance, the trigeminal sensory and vestibular ganglia neurons of these animals are intact.

The major phenotypes of the GDNF null mice are the arrested development of the metanephric kidney and the enteric nervous system. The GDNF -/- mice have regularly bilateral renal agenesis (Pichel et al., 1996). Also the GDNF +/- heterozygous animals frequently show renal abnormalities such as unilateral aplasia or dysplasia. Morphologically distinct metanephric blastema is present in the mutant embryos, and the primary defect is the inability of the ureter to bud from the Wolffian duct.

The second major functional phenotype of the GDNF -deficient mice is the peristaltic defect of the gut. This is due to the total absence of neurons in the parasympathetic plexuses of the gastrointestinal tract below the cardiac stomach. Despite the strong expression of GDNF in the esophagus, the esophageal neurons develop normally. At this point it is not clear whether GDNF is needed for the migration of the neural crest cells to the gastrointestinal tract or whether it functions as a supportive or differentiation factor for the enteric neurons.

Ret receptor tyrosine kinase

As shown in this study, the functional receptor for GDNF is the Ret tyrosine kinase (I). The *Ret* (REarranged during Transfection) proto-oncogene was originally discovered by Takahashi and colleagues who showed a novel gene rear-

rangement and oncogenic activation in a transfection assay of NIH 3T3 cells with lymphoma DNA (Takahashi et al., 1985). The activated gene was then found to code for a novel tyrosine kinase that has an extracellular ligand-binding domain with two cadherin-like repeats, a hydrophobic transmembrane region and a cytoplasmic domain with an intrinsic tyrosine kinase activity. The Ret gene has 21 exons that span more than 60 kb of genomic DNA and the mRNA can be alternatively spliced to produce up to ten protein isoforms (Myers et al., 1995). The Ret gene has been assigned to the human chromosome 10q11.2 (Ishizaka et al., 1989).

Ret in development

Ret is expressed during embryogenesis on various neuronal subsets of the central and peripheral nervous system, including the nervous plexuses of the entire intestinal tract (Pachnis et al., 1993; Watanabe et al., 1997). Outside the nervous system, Ret is expressed in the Wolffian duct, budding ureter and later in the tips of the renal collecting tubules. The expression pattern of the receptor is in accordance with its ligand distribution, because in most cases Ret and GDNF are expressed in adjacent cells or tissues. In addition to the parallel expression patterns, a further clue to the possible interaction between GDNF and Ret came from the knockout animals. Like GDNF null mice, also the homozygous Ret-deficient mice are born alive, but then die during the first postnatal day because of renal aplasia or hypodysplasia (Schuchardt et al., 1994). They also lack enteric nervous plexuses in both their small and large bowels. The only major abnormality in other areas of the nervous system is the lack of the superior cervical ganglia (Schuchardt et al., 1994) that are partially affected in the GDNF null mice, as well.

Ret signal transduction

The biochemistry downstream of Ret has been difficult to approach because the ligand has not been available for use, until recently. Most of this work has therefore been done by using the constitutively active mutant Ret molecules from the cancer syndromes, or by using chimeric proteins, like the EGFR ligand binding part in front of the Ret catalytic domain (Santoro et al., 1994). Like other tyrosine kinases, upon ligand binding Ret autophosphorylates its tyrosine domains that then serve as docking sites for Src-homology 2 (SH2) domain containing target molecules activating different intracellular pathways. Ret associates with Shc, Grb2, phospholipase C gamma, Crk, Nck, and Grb10 proteins (Pandey et al., 1995; Bocciardi et al., 1997; Ohiwa et al., 1997) and at least one major pathway shown to be activated by Ret is the Ras-MAP kinase pathway (Ohiwa et al., 1997; Worby et al., 1998). Phosphopeptide maps have showed that the autophosphorylation of Ret, found in multiple endocrine neoplasia (MEN)2B, differs from that of the wild-type and MEN2A Ret proteins (Liu et al., 1996), suggesting that also the targets downstream of them may differ (Zhou et al., 1995). Some of these targets like paxillin have now been identified (Bocciardi et al., 1997).

Ret in human diseases

The gene for Ret is a special disease gene, since at least five different diseases can be the result of its mutations. As was shown by Takahashi et al. in 1985, Ret is able to transform cells, when translocated.

Similar gene translocations have been found in human papillary thyroid carcinomas (Grieco et al., 1990), where the tyrosine kinase coding domain of Ret gene is fused with the 5' sequence of a gene that is expressed in the follicular cells leading to ectopic Ret activation. Ret gene mutations have been described in the MEN2A and MEN2B hereditary cancer syndromes, and in medullary thyroid carcinoma (Donis-Keller et al., 1993; Mulligan et al., 1993). In these diseases, the oncogenic mutations constitutively activate the phosphorylation of the receptor. For example, the MEN2A mutation changes one cysteine residue in the extracellular domain of the molecule leading to the dimerization of the receptor without ligand binding (Santoro et al., 1995). All the mutations found in the Ret gene have been recently reviewed (Edery et al., 1997). The cancer syndromes MEN2A, MEN2B and familiar medullary thyroid carcinoma are often referred to as neurocristopathies, since the affected cells originate from the neural crest. An additional neurocristopathy is the Hirschsprung's disease or aganglionic megacolon, which is often caused by an inactivating Ret mutation, as described later.

GDNF family receptor alphas

The initial crosslinking experiments with GDNF (Trupp et al., 1995) showed saturation of GDNF binding in embryonic chick sympathetic neurons at 1-5 x 10^{-9} M, that is weaker than the interaction between most other growth factors and their receptors, like NGF and Trk (Kaplan et

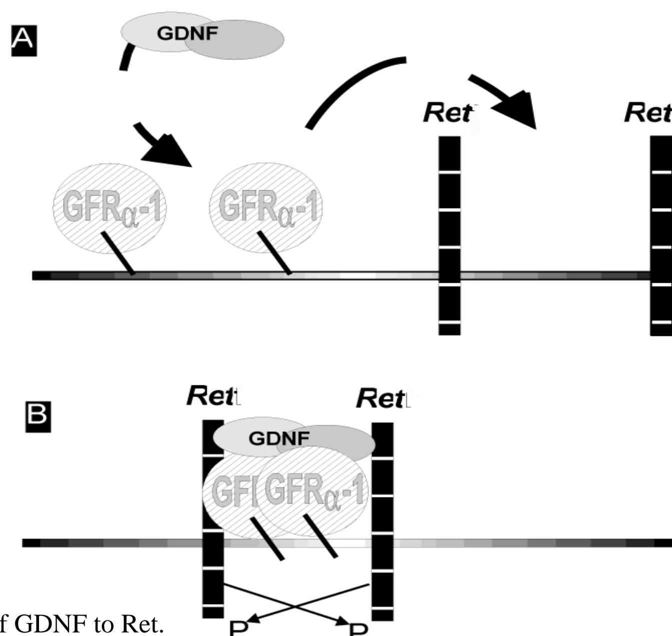


Figure 3. Binding of GDNF to Ret.
A. GDNF dimer binds to two molecules of GFRa-1 and this complex binds to Ret that dimerizes.
B. The dimerization of Ret causes the autophosphorylation of the tyrosine residues of the two subunits.

al., 1991). Later it was found that the high affinity GDNF binding to Ret is mediated through a glycosylphosphoinositol-linked protein termed the GDNF family receptor alpha (GFRa, first described as GDNFR-a) (Jing et al., 1996; Treanor et al., 1996). The dissociation constant (K_d) between GDNF and GFRa was then shown to be approximately 2x 10^{-12} M in the transfected 293T cell line. As illustrated in Figure 3, a dimer of GDNF first binds to two molecules of GFRa and the GDNF-GFRa complex binds to and activates Ret. It has now become apparent that different cell types show different affinities to GDNF, which is most likely due to their different expression pattern of receptors.

The family of GFRas has rapidly expanded, and comprises at the moment three members from GFRa-1 to GFRa-3 (Jing et al., 1996; Treanor et al., 1996; Baloh et al., 1997; Jing et al., 1997; Klein et al., 1997; Masure et al., 1998; Naveilhan et al., 1998; Worby et al., 1998, II). GDNF and NTN seem to prefer GFRa-1 and GFRa-2, respectively, although they are able to bind and activate Ret by using either one of these two a-receptors (Baloh et al., 1997; Jing et al., 1997). At this stage, the ligand of GFRa-3 is not known, but it seems not to be PSP. The expression patterns of the GFRas are both overlapping and complementary with each other, the ligands and with the expression of Ret, which suggests that the biological functions of the GDNF family can be regulated in very many ways, like the choice of the receptor or the concentration of the factors.

DEVELOPMENT OF THE ENTERIC NERVOUS SYSTEM

The enteric nervous system (ENS) derives from neural crest precursor cells that migrate and colonize the wall of the intestinal tract during embryogenesis (Le Douarin and Teillet, 1973). Both experimental and descriptive data show that three different regions of the neural crest - vagal, truncal and sacral - contribute to the precursors of intestinal neuroblasts and Schwann cells, and that the different regions of the neural crest give rise to the neuroblasts colonizing distinct regions of the gut (Serbedzija et al., 1991; Durbec et al., 1996; Gershon 1997). The migration begins with an outgrowth of vagal neural crest precursor cells, which use the ventral pathway and migrate through the somites. The first neuroblasts are detectable in the proximal foregut of mice at embryonic day 9.5 (Em9.5) and then proceed in a cephalo-caudal manner (Kapur et al., 1992). The vagal progenitors colonize the entire gut reaching the terminal hindgut by Em13.5, while the progenitors arising from the truncal and sacral regions will migrate only to the rostral foregut and postumbilical bowel, respectively (Gershon 1997).

Neural crest chimeras between chicken and quail have revealed that the precursors for the intestinal nervous plexus migrate from the neural crest as single cells or small cell clusters and, in the front of the migration, intermingle with the mesenchymal cells of the intestinal wall (Le Douarin and Teillet, 1973). They later become redistributed in the presumptive Auerbach (intermuscular) and Meissner (submucosal) plexuses, when the muscular layers of the gut begin to differentiate. The migrating neuroblasts are still capable of differentiating into various types of neural cells, as shown by the tissue transplantation experiments of various researchers (Rothman et al., 1990; Lo and Anderson, 1995). The developmental options of the precursor cells become progressively restricted during the innervation process when the cells migrate in a certain microenvironment, but the mechanisms determining the fate of the intestinal nerves are not yet known.

GDNF signaling pathway in the developing gut

The mice deficient for GDNF lack the enteric nerves distal to the cardiac stomach (Pichel et al., 1996), which indicates that GDNF is crucial at some point during the enteric nervous system development. The function of GDNF in the enteric innervation is supported by the expression data. By *in situ* hybridization, RNase protection assay and RT-PCR studies GDNF mRNA has been found in the intestinal muscle layers from esophagus to rectum in embryonic mice and rats (Choi-Lundberg and Bohn, 1995; Hellmich et al., 1996; Suvanto et al., 1996). In addition, at least rat stomach and small intestine continuously express GDNF mRNA after birth to adulthood (Choi-Lundberg and Bohn, 1995).

Ret, the functional receptor for GDNF, is expressed in the migrating enteric neuroblasts already during their early development (Pachnis et al., 1993). These enteric neuroblasts in Ret-deficient mice undergo apoptosis soon after they reach the level of the duodenum (Durbec et al., 1996). Because Ret- and GDNF-deficient mice show similar defects in ENS, GDNF has been thought to be a signal conducting the ENS precursor cells to the right direction and position in the intestine.

Prior to this study, no data have been reported for the mRNA distribution of GDNF in human intestine. However, GDNF protein has been studied in normal colon as well as in affected and healthy colonic segments of the aganglionic megacolon i.e. Hirschsprung's disease patients by immunohistochemistry and immunoassay (Bär et al., 1997). GDNF-like immunoreactivity was found in the neural fiber-like structures in the gut, and there were no differences in GDNF levels between the ganglionic and aganglionic segments of Hirschsprung's disease patients. The ganglionic expression of Ret protein has been demonstrated in human embryonic and newborn intestine by immunohistochemical studies (Martucciello et al., 1995; Tam et al., 1996). In addition to GDNF and Ret, GFRα-1 and GFRα-2 are expressed in mouse gut, but no data on the alpha receptors in human development had been previously reported.

NEUROTROPHIC FACTORS AND THEIR RECEPTORS IN HUMAN DISEASES

Neurotrophic factors and their receptors are important developmental and neuronal molecules and therefore good candidates to be responsible for different developmental and neurodegenerative diseases. In Hirschsprung's disease, approximately 30 percent of the patients have mutations in the Ret gene. GDNF is a strong survival factor for the dopaminergic neurons that degenerate in Parkinson's disease. Meckel syndrome is a severe brain malformation that had been mapped to the same region with the p75NTR gene. These diseases will now be discussed in more detail.

Hirschsprung's Disease

Clinical picture

Hirschsprung's disease patients suffer from severe obstipation that usually, but not always, manifest shortly after birth (Hirschsprung 1888). The absence of ganglia in the entire length or various segments of the large bowel characterizes the disease, also termed aganglionic megacolon. In addition to the lack of enteric ganglia, a highly increased density of cholinergic nerve fibers is seen in the colonic mucosa. The diagnosis is made by a combination of tests, such as anomanometry, radiological examinations and histological, histochemical or immunohistochemical analyses of the affected region of the colon (Molenaar et al., 1989; Martucciello et al., 1998). The treatment of Hirschsprung's disease is the surgical removal of the aganglionic segment, which gives good or fairly good result in most of the patients (Langer et al., 1996; Heikkinen et al., 1997). The choice and result of different surgical protocols depends on the age of the patient, severity of the disease, including the length of the affected gut, and the biggest postoperative problems are persistent constipation and enterocolitis (Langer et al., 1996).

Genetics of Hirschsprung's disease

The variable clinical manifestations and inheritance of Hirschsprung's disease suggest that it is a multifactorial disorder. Most cases are sporadic, but both autosomal recessive and dominant modes of inheritance have been reported (Badner et al., 1990). It has been assumed that cases with extensive defects in the intestinal innervation are more likely inherited. An increased sex ratio (3 to 5 males: 1 female) and an elevated risk of siblings (4%), as compared with the population incidence of Hirschsprung's disease, have been reported (Badner et al., 1990).

Aganglionic megacolon is not always found as an isolated defect but is a frequent finding, e.g. in Down syndrome (Passarge 1967). Several other more or less linked syndromes include cartilage-hair hypoplasia, the Smith-Lemli-Opitz syndrome type II, Waardenburg-Hirschsprung disease, piebaldness and primary central hypoventilation syndrome (Ondine's curse) (Passarge 1967; O'Dell et al., 1987; Badner et al., 1990).

GDNF signaling in Hirschsprung's disease

In contrast to the sporadic cancers and cancer syndromes with mutations constitutively activating Ret, approximately 25 to 30 percent of patients with Hirschsprung's disease show mutations inactivating the Ret signaling. These mutations vary from missense or nonsense point mutations to deletions (Pasini et al., 1996; Edery et al., 1997) and they result in the inactivation of the tyrosine kinase or ligand binding domain or in the perturbance of the membrane transport of the receptor (Edery et al., 1994; Angrist et al., 1995; Attie et al., 1995; Pasini et al., 1995; Carlomagno et al., 1996; Ito et al., 1997).

An interesting question is, why do the Hirschsprung's disease patients only rarely have renal malformations? One explanation is that all Ret gene mutations in Hirschsprung's disease described until now have been heterozygous. This is in accordance with the lack of a kidney phenotype in the Ret heterozygous mice. The reduced number of functional Ret receptors in Hirschsprung's patients may not be enough to affect renal morphogenesis in man, but is enough to perturb morphogenesis in a less redundant organ, the intestine. Also, there is no correlation between the site and size of the mutation in Ret gene and the extent of colonic aganglionosis (Attie et al., 1995). This unexpected finding may reflect modulation of the clinical phenotype by unknown genes. Some mutations in the Ret gene, in particular those that block the transport of the receptor to cell surface certainly reduce the amount of active Ret receptors to half. Some other mutant and inactive Ret receptors are transported to the cell surface and could act as dominant-negative inhibitors of the wild-type receptor (Carlomagno et al., 1996; Ito et al., 1997).

Soon after the identification of GDNF as a ligand for Ret, many groups analyzed the GDNF gene in a number of patients with Hirschsprung's disease. Only seven patients with five different heterozygous variants in the GDNF gene have been found in materials including more than 300 patients (Angrist et al., 1996; Ivanchuk et al., 1996; Salomon et al., 1996). Some of these variants were also found in healthy individuals and in one family the father was homozygous for the same variant as his affected child, but did not have the Hirschsprung's disease himself (Salomon et al., 1996). It seems that the question of the genetics and the consequences of the GDNF mutations is complicated and more genes may be involved simultaneously as the variations in both alleles of one gene did not lead to disease in the above mentioned family. Furthermore, biochemical analyses of these GDNF mutations in the receptor kinetics have not been performed.

Some alterations in Hirschsprung's disease have been found in the gene coding for GFRα-1, GFRα-2 (Myers et al., 1998) and NTN (Salomon et al., 1998), but it looks like there may not be any clear disease causing mutations.

Endothelin signaling and other genes in Hirschsprung's disease

Several genes in endothelin signaling have turned out to be involved in the pathogenesis of Hirschsprung's disease. Endothelins (EDN1, EDN2 and EDN3) form a family of small 21 amino acid peptides that were discovered as potent

smooth muscle contractors on vascular endothelia (Yanagisawa et al., 1988; Inoue et al., 1989). Mature endothelins are processed from a precursor protein by the endothelin converting enzyme 1 (ECE1) and they mediate their effects through two endothelin receptors (EDNRA or EDNRB) that belong to the family of G-protein-coupled heptahelical receptors. EDNRB is expressed in brain, kidney, lung, heart and endothelia, but the protein is also found in the human colon, i.e. the myenteric plexus, mucosal layer, ganglia and blood vessels of the submucosa (Inagaki et al., 1991; Sakamoto et al., 1991).

EDNRB was the affected gene in a previously mapped susceptibility locus of a recessive form of Hirschsprung's disease (Puffenberger et al., 1994) and a specific missense mutation W276C predisposed to the disease. This mutation was first found in a consanguineous pedigree, and it was dosage sensitive. W276C homozygotes and heterozygotes had a 74% and a 21% risk of developing Hirschsprung's disease, respectively. Other heterozygous mutations both in syndromatic and isolated Hirschsprung's disease as well as in non-consanguineous populations have later been found (Attie et al., 1995; Amiel et al., 1996; Auricchio et al., 1996). The next disease gene for Hirschsprung's disease was endothelin 3 (EDN3), the gene encoding the ligand for EDNRB (Edery et al., 1996; Hofstra et al., 1996). These mutations are homozygous and were first found in a syndrome resulting in a widespread developmental failure of neural crest derivatives, the combined Waardenburg-Hirschsprung disease (Shah-Waardenburg syndrome) with aganglionic megacolon, sensoryneural hearing loss and pigmentary anomalies. A heterozygous EDN3 mutation has been found in isolated Hirschsprung's disease (Bidaud et al., 1997) and one ECE1 mutation has also been described recently (Hofstra et al., 1998).

Several mouse models have helped the search for Hirschsprung's disease genes and the pathogenesis of aganglionosis. In addition to the GDNF and Ret knockout mice, there are several natural mutant lines with failures in the neural crest cell migration. They show Hirschsprung –like aganglionic megacolon and, in some lines, also the absence of melanocytes in the skin. The alleles of *lethal spotted* (*ls*) and *piebald lethal* (s^l) (Lane 1966) in natural mutant mouse strains code for the endothelin 3 (Baynash et al., 1994) and endothelin receptor b (Hosoda et al., 1994), respectively. A third strain of *Dominant megacolon* mice has a mutation in the Sox 10 gene that encodes for an SRY-like transcription factor (Southard-Smith et al., 1998). Very recently, patients with Shah-Waardenburg syndrome with aganglionic megacolon, pigmentary anomalies and sensoryneural deafness, were shown to have a mutation in the SOX10 gene (Pingault et al., 1998).

Parkinson's Disease

Parkinson's disease is a common neurodegenerative disorder with a prevalence of approximately 1-2% in the population over 50 years of age. The patients suffer from resting tremor, bradykinesia, rigidity and postural instability that is caused by the progressive loss of dopaminergic neurons in the substantia nigra. The pathogenesis of Parkinson's disease is unclear, although several hypotheses exist. The neuronal

loss has been proposed to occur because of oxidative stress due to free radicals, environmental factors such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), amino acid or calcium toxicity (Temlett 1996).

The first identified Parkinson's disease gene seems to be alpha-synuclein, coding for a presynaptic protein that is defective in a familial form of the disease (Polymeropoulos et al., 1997). A second susceptibility locus has been found in the chromosome 2p13 (Gasser et al., 1998) and a gene responsible for a rare recessive form of juvenile parkinsonism has been identified (Kitada et al., 1998). These genes are unlikely to play a common pathogenic role in Parkinson's disease since no mutations have been found in the sporadic forms of the disease. The normal functions of alpha-synuclein and the novel gene, parkin, are not known. Many efforts have been performed to identify gene mutations in Parkinson's disease, but these have led only to exclusions of some candidate genes, like superoxide dismutase and catalase (Parboosingh et al., 1995).

GDNF in Parkinson's disease

The level of GDNF mRNA in the adult CNS seems to be very low in both Parkinson's patients and controls (Hunot et al., 1996), and the GDNF protein levels or even its function in the adult human brain are not known. Furthermore, the dopaminergic cells of Parkinson's disease patients are difficult to study because most of them are lost during the disease process.

Most of the studies of GDNF in Parkinson's disease have been performed in animals. There are multiple ways of creating animal models for these studies by damaging the midbrain dopaminergic neurons in rodents or primates. The toxins 6-hydroxydopamine (6-OHDA) (Simon et al., 1974) or 1-methyl-4phenyl-1,2,5,6-tetrahydropyridine (MPTP) (Heikkila et al., 1984) have been mostly used to create parkinsonism that especially in primates resemble the human disease in great detail. These animal models have then been used to look for and study the molecules that would protect, support or even cure the symptoms. In these kinds of experiments, GDNF has proved to be the most potent neuroprotective factor *in vivo* by preventing and rescuing the dopaminergic neurons from degeneration and relieving the symptoms even after neuronal damage (Lin 1996; Lapchak et al., 1997)) (Table 5). The level of GDNF seems to be critical for the survival of the neurons, which suggests that GDNF could be used in the prevention of the progression of Parkinson's disease.

Meckel syndrome

Meckel syndrome is a lethal autosomal recessive syndrome characterized by an occipital meningoencephalocele, large multicystic kidneys, fibrotic changes of the liver and postaxial polydactyly (Salonen and Paavola, 1998). The clinical features are variable and include additional anomalies, like cleft palate, congenital heart defects, club feet and ocular anomalia (Salonen 1984). The prevalence of Meckel syndrome in Finland is 1:9000 (Salonen and Norio, 1984), but it varies in different populations from 1:1300 in Gujarati Indians to 1:50 000 in Israeli Jews

Table 5. GDNF rescue studies in animal models for Parkinson's disease.

Lesion type	Rescue method	Result	References
6-OHDA in rat: injections uni- or bilaterally	GDNF-injections intranigrally or GDNF adenoviral transfection	dopaminergic neurons restored, symptoms decreased	(Hoffer et al., 1994; Kearns and Gash, 1995; Sauer et al., 1995; Bowenkamp et al., 1997; Choi-Lundberg et al., 1997)
Axon transection in rat	Daily intranigral GDNF-injections	dopaminergic neurons restored	(Beck et al., 1995)
MPTP in mouse: subcutaneous injections	GDNF-injection intrastriatally or intranigrally before or after MPTP	dopaminergic neurons restored	(Tomac et al., 1995)
MPTP in rhesus monkey: carotid artery infusion	GDNF- injection intracerebrally, intracaudally or intranigrally every four weeks	dopaminergic neurons restored, symptoms decreased	(Gash et al., 1996)

(Salonen and Paavola, 1998). Although Meckel syndrome is clearly a recessive disorder, some reports suggest also a milder heterozygous phenotype in the relatives of the patients (Salonen and Norio, 1984; Gulati et al., 1997).

The gene causing Meckel syndrome is not known. In 17 Finnish families the gene locus was mapped to chromosome 17q21-q24 (Paavola et al., 1995) to the close vicinity of the p75NTR gene. This region has been excluded in some other families (Paavola et al., 1997; Roume et al., 1997), suggesting genetic heterogeneity.

Meckel syndrome is recognized often in rutine mid-gestation ultrasound screening and the diagnosis is verified by autopsy after the termination of the pregnancy. The high-risk families that already have had affected children can be scanned at 11-12 weeks of pregnancy by vaginal ultrasound (Sepulveda et al., 1997). In families with established linkage to 17q21-q24, the prenatal diagnosis of Meckel syndrome can be done by DNA analysis.

The pathological anatomy and histology of Meckel syndrome patients have been thoroughly studied in different tissues and the similarity of findings in multiple organs (Rapola and Salonen, 1985; Rapola 1989) suggest a failure in inductive interactions during the development. The genes coding for developmental factors expressed in the affected tissues are therefore interesting Meckel syndrome candidate genes. One of these candidates mapped in 17q21-q24, the p75NTR gene, is expressed in the brain, kidney, testis and ovaries and is also critically involved in nephrogenesis (Buck et al., 1987; Sariola et al., 1991).

STRATEGIES FOR THE IDENTIFICATION OF DISEASE GENES

There are many possible ways to search for genetic associations and gene alterations in diseases. Chromosomal abnormalities can be visualized with a microscope from a metaphase cell preparation, but in most cases the alterations are only minor and need molecular genetic methods to be found (Foroud 1997). In a classical disease gene mapping project, the locus of an inherited disease gene is first assigned to a chromosomal region with microsatellite markers, after which the search for the gene itself is started. The disease gene is then identified by narrowing the size of the target region, by positional candidate approach or systematically by physical mapping and analyzing all the genes in that area.

Candidate genes

Most diseases are not inherited, or the inheritance pattern is so complicated that it cannot be easily observed. In these sporadic or complex and multigenic diseases, the positional cloning method is less efficient or impossible, and the identification of disease genes is often based on a candidate gene approach that is based on the functional and expression data of the gene. The possibility of finding a disease gene by this approach depends on the information that is available of the candidate gene that can be compared with the pathology or pathophysiology of the human disease. The optimal situation is if: 1) The structure, function and expression of the candidate gene are known. 2) There is an animal model for the disease and/or mice with targeted disruption of the candidate gene. 3) The gene is conserved during evolution. The candidate gene approach is also powerful in the search for inherited disease genes, when the disease locus has been assigned by positional cloning.

Finding the candidate genes will become easier in the future, with the advance in the Human Genome Project (http://www.nhgri.nih.gov/HGP/). This worldwide research effort is to characterize the structure of the human genome by sequencing and mapping. The project is also to characterize the genomes of yeast, worm, fruit fly and mouse parallel with the human studies, which gives more information of the function and evolution of the genes.

Mutation identification

In some diseases, such as cancer, the usual genetic event is a translocation or a deletion of several hundreds to millions of base pairs which can be identified by comparative genomic DNA hybridization or in Southern or Northern hybridization from DNA or RNA, respectively, with a candidate cDNA as a probe. In many inherited and sporadic developmental disorders, the disease causing mutation changes only one nucleotide and must therefore be detected by using more sensitive methods (Cotton 1997; Aaltonen, 1998).

The methods for detecting point mutations are usually based on polymerase chain reaction (PCR) and they can be divided into three categories: 1) Direct sequencing. 2) Aberrant migration of the mutant PCR product. 3) Enzymatic cleavage in a mismatch heteroduplex molecule. When choosing which method to use, the advantages and disadvantages of each alternative must be weighed (Cotton 1997). The direct sequencing of the PCR product is most informative, but it is also the slowest and most expensive of the three methods. This is likely to change in the future when the DNA "chip" technique is advanced (Southern 1996). The methods monitoring the aberrant migration of the mutant PCR product amplified from the patient template include single strand conformation polymorphism (SSCP) (Orita et al., 1989), denaturing gradient gel electrophoresis (DGGE) (Myers et al., 1987) and heteroduplex analysis (HA) (Nagamine et al., 1989). These methods are fast but they are not 100% sensitive and the mutation has to be identified by sequencing the samples with altered mobility. These same disadvantages are found also in the third category of the mutation detection methods, although they are more sensitive, allow the screening of longer fragments and give a rough estimate of the location of the mutation in the sample. These approaches include the RNase A cleavage, chemical cleavage, enzyme mismatch cleavage and cleavase fragment length polymorphism (CFLP) methods (Cotton 1997).

Mutation-disease correlation

The consequences of a certain sequence variant or its role in a disease pathogenesis is sometimes difficult to determine. A nonsense or frameshift mutation that leads to a truncated protein, most likely disrupts the gene function, but a gene with a missense point mutation can be totally functional. The importance of a certain amino acid to the gene function can be estimated by comparing the conservation of the residue between different species during evolution. In inherited diseases, a strong evidence for a causative genetic change is the segregation of the mutation with the disease phenotype and the absence of the variation in controls. Functional test for the mutant gene would be most informative to find out the consequence of the variation, but they are often not feasible.

The allele frequency of the identified variation can be determined in a large number of normal and affected samples by sequencing, restriction fragment analysis or for example by "minisequencing" pools of DNA from a number or individuals (Syvänen et al., 1993). However, in more complicated genetics where a disease is multigenic, has a low penetrance or may display several modes of inheritance one has to be careful with the interpretation of allele frequency analyses. As has been seen in the genetics of Hirschsprung's disease, the segregation of the disease with the mutation does not necessarily prove the mutation to be causative for the disease. On the other hand, a variation can be a disease associated mutation even without being necessary for the disease phenotype or sufficient to cause the disease alone (Salomon et al., 1996). These genes could be called disease-associated with major or minor modifying sequence variants - factors that we are going to run into now when the genetics of complex disorders is starting to be revealed (Hofstra et al., 1997).

AIMS

The aims of this thesis were to study the biology and pathology of the genes involved in neurotrophic signaling. We wanted to characterize the function of these developmentally important factors in more detail and to study their possible roles in diseases.

The specific aims of this work were:

1. To identify and characterize receptor(s) for GDNF
2. To study the expression and function of GDNF and its receptors in the developing gut and kidney
3. To search for mutations in the GDNF gene in diseases that are closely linked to its function: Hirschsprung's disease, renal agenesis and Parkinson's disease.
4. To search for mutations in the p75NTR gene in Meckel syndrome

MATERIALS AND METHODS

Cloning of human GDNF

The cDNA for human GDNF was cloned by RT-PCR from the fetal brain RNA and ligated to TA cloning vector (Invitrogen) according to manufacturer's protocol. Several clones were grown, isolated and sequenced in both directions.

Cloning of human and rat GFRa-2 cDNA

Several human clones similar to GFRa-1 were found in the EST database search. The rat cDNA was cloned from adult rat hippocampus library and the 5'-end of the human cDNA was amplified by PCR from human fetal cDNA. The cloning procedures are described more in detail in (II).

Probes

The probes for *in situ* and Northern hybridizations of human samples were synthesized from cDNA inserts of human GDNF (636 bp), GFRa-1 (2537 bp, a kind gift from Dr. G.Fox, Amgen), GFRa-2 (1490 bp) and mouse ret (714 bp) clones. The probes for FISH were the human 1490 bp GFRa-2 cDNA and a mouse 10 kb genomic fragment containing the initiation ATG-codon.

Fluorescent in situ hybridization

The fluorescent in situ hybridization of metaphase chromosome lymphocytes was performed using the 1490bp human cDNA and 10kb mouse genomic probes as described in (II).

Northern analysis

The detection of the GFRa-2 mRNAs from different human tissues was done by hybridizing radiolabeled GFRa-2 probe to Human and Human Fetal Multiple Tissue Northern Blot filters as described in (II).

Growth factors and antibodies

Human recombinant GDNF was purchased from Promega and PeproTech Inc. Baculovirus -produced rat GDNF was a gift from Dr. Carlos Ibáñez. The antibodies to Ret and phosphotyrosine were from Santa Cruz and Transduction Laboratories, respectively.

Receptor tyrosine phosphorylation assay

The immunoprecipitation and Western blotting of the Ret protein with anti-Ret and anti-phosphotyrosine antibodies are described in detail in (I) and (II).

Sample tissues

The tissues of patients were taken from autopsy or blood samples with the full ethical permission of the patents or the hospital in question (Hospital for Children and Adolescents in Helsinki, Karolinska Institutet in Stockholm and Turku University hospital). The tissues were treated as described in (III-V).
In the animal experiments tissues from Sprague-Dawley and Wistar rat and mouse embryos were used. In these experiments, the animals were mated overnight and the next day was defined as embryonic day 0 (E0).

p75NTR immunohistochemistry

Mouse antibodies to human p75NTR (Boehringer) were used at 1:10 dilution to stain acetone-fixed cryosections of placenta, kidney and spleen of 3 Meckel syndrome patients and 3 age-matched controls (V). The staining was visualized by FITC-conjugated antibodies to mouse IgG.

In situ hybridization

In situ hybridization was performed as described (Wilkinson and Green, 1998), with slight modifications (Kallunki et al., 1992) that are described in (III and IV).

RT-PCR

The extraction of polyA mRNA from the fetal and newborn colon, and the RT-PCR protocol are described in more detail in (III).

Cell and organ cultures

The tissues for organ cultures were microdissected and cultured in Trowell-type dishes as described in (IV).

SSCP analysis

SSCP analysis with the radiolabeled PCR fragments was performed as described (Orita et al., 1989) with some modifications (Suomalainen et al., 1992).

Mutation search by sequencing

The sequencing of direct PCR fragments and their cloned inserts of patient samples were done in the DNA synthesis and sequencing core-facility of the Institute of

Biotechnology. Different samples were sequenced either in ALF or ABI sequencers according to the current protocol for either solid-phase or cycle sequencing.

Minisequencing

The mutation frequency analysis by minisequencing was performed as described (Syvanen et al., 1993).

Radiation hybrid mapping

Radiation hybrid mapping panel provided by Research Genetics was utilized to order the genes of NGFR and HOXB6 as well as the polymorphic microsatellite markers D17S806, D17S1607, D17S957, and D17S807 as described in (V). Radiation hybrid mapping data was analyzed using multipoint maximum likelihood approach and the FORTRAN programs RH2PT and RHMAXLIK from the package RHMAP version 2.01 (Boehnke et al., 1991). In multipoint analysis we carried out branch and bound strategy assuming equal fragment retention as well as allowing centromeric effect. Breakage probabilities theta were converted to additive distances d (cR) according to the formula d = -ln(1-theta) (Cox et al., 1990).

CFLP analysis

CFLP mutation analysis of the GDNF gene PCR products of the Parkinson's disease patients was done according to manufacturer's (Boehringer Mannheim) protocol.

RESULTS AND DISCUSSION

Ret mediates GDNF signal in mesoderm induction of the *Xenopus* blastula animal caps (I)

The animal cap cells of blastula-stage *Xenopus* embryo give rise to epidermal tissue, if untouched. However, the activation of the mitogen activated protein kinase (MAPK) is sufficient and necessary to induce the mesoderm in these cells (Umbhauer et al., 1995). The injection of *in vitro* synthesized RNA of a constitutively active Ret receptor (Ret ^{C634R}) that signals through the MAPK led to the mesoderm induction, while wild type Ret or GDNF RNA injections alone did not. The mesoderm induction, defined by morphological, histological and molecular markers was however gained by co-injecting the mRNAs of wild type Ret and GDNF, suggesting that GDNF activates Ret signaling. This result was specific for Ret, as another tyrosine kinase Sek1, co-injected with GDNF mRNA, did not induce the mesoderm. Further support of the GDNF signaling through Ret was obtained by chemical crosslinking studies.

In addition to the biological assay described above, several groups simultaneously reported the interaction between GDNF and Ret (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996; Vega et al., 1996). Also another receptor for Ret, GFRa-1 (previously GDNFRa) was iden-

tified and shown to be the high-affinity binding co-receptor for GDNF (Jing et al., 1996; Treanor et al., 1996).

The finding of tyrosine kinase receptor Ret as a functional receptor for GDNF was unexpected, because all previously known TGFb family members bind to serine-threonine kinases (Josso and di Clemente, 1997). These results do not rule out the possibility that GDNF could interact with alternative, serine-threonine kinase receptors.

Cloning and characterization of the GFRa-2 gene (II)

The initial cloning of GFRa-2 (formerly GDNFRb) was done by searching the Expressed Sequence Tag (EST) database with the sequence of GFRa-1 as a probe, which identified eight human sequences from six different clones. These clones were sequenced to form a contig of 1032 bp of human GFRa-2 cDNA. The rat cDNA was then cloned from the rat hippocampal cDNA library and the 5'end of the human gene was amplified from human fetal brain cDNA.

The GFRa-2 gene was found to be a close homolog to GFRa-1 with 48% identity at the predicted amino acid level. All the cysteine residues are conserved which suggests a similarity in the tertiary structure. GFRa-2 has a signal sequence, three N-glycosylation sites and a putative glycosyl-phosphatidylinositol anchor site similar to GFRa-1. The loci of the GFRa-2 gene were assigned to the human chromosome 8p21-p22 and to a corresponding region in the mouse chromosome 14D3-E1 by fluorescent *in situ* hybridization (FISH).

Expression and function of GFRa-2 (II)

By northern hybridization we found two major GFRa-2 transcripts of 3.5 and 2.9 kb that were expressed in human adult brain, intestine, placenta and fetal kidney. Additional transcripts of 7.5 kb and 1.4 kb were also visible in placenta and testis, respectively. The *in situ* hybridization of GFRa-2 and GFRa-1 showed non-overlapping mRNA distribution in several organs of the rat E17 embryo. GFRa-2 mRNA was seen in the capsule and cortex of adrenal gland, the undifferentiated nephrogenic mesenchyme of the kidney and vaguely in the enteric plexuses of the small intestine. GFRa-1 mRNA was seen in the adrenal medulla, in the tips of ureter buds and strongly expressed along the gastrointestinal tract where GFRa-2 was not or was only weakly expressed.

The ability of GFRa-2 to mediate GDNF-induced activation of Ret was analyzed by transfecting the COS-7 cells with the cDNAs encoding GFRa-1, GFRa-2 and Ret in different combinations. The transfection of either GFRa-1 or GFRa-2 in combination with Ret resulted in Ret phosphorylation after GDNF treatment, which indicated that GDNF can bind to and signal through a Ret/GFRa-2 complex.

Several groups simultaneously reported the GFRa-2 gene with different names. The name of GFRas is the consensus of the nomenclature committee (GFR-alpha nomenclature committee 1997) that ceased the use of TGF-b related neurotrophic factor receptor 2 (TrnR2) (Baloh et al., 1997), Ret ligand 2 (RetL2) (Sanicola et al., 1997), neurturin receptor

a (NTNRa) (Buj-Bello et al., 1997; Klein et al., 1997) and GDNF receptor b (GDNFRb) (II).

These studies confirmed the function of GFRa-2 in Ret activation and suggested that the preferred ligands for GFRa-1 and GFRa-2 are GDNF and NTN, respectively (Baloh et al., 1997; Buj-Bello et al., 1997; Klein et al., 1997), although both GFRa-1 and GFRa-2 can bind both ligands. The mechanism of GFRa-1 and GFRa-2 to mediate Ret activation is different, since GFRa-1 first binds to GDNF and then to Ret (Jing et al., 1996), but GFRa-2 does not form complexes with GDNF in the absence of Ret (Sanicola et al., 1997).

Distribution of GDNF and its receptors in the developing human gut (III)

By *in situ* hybridization we found that GDNF mRNA expression was abundant in the muscularis mucosae of both fetal

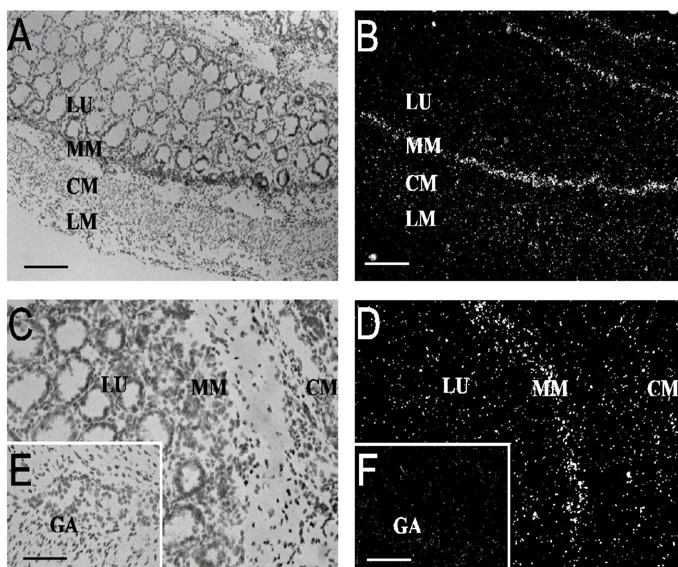
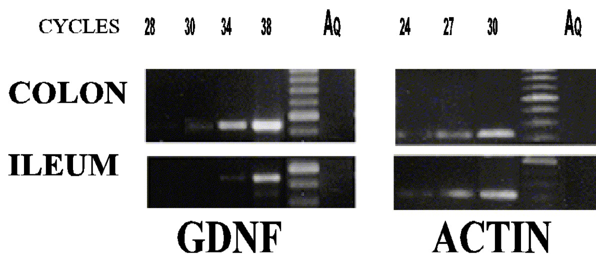


Figure 4.
Upper: Bright (A,C,E) and dark (B,D,F) field images of GDNF *in situ* hybridization showing mRNA in the muscularis mucosae (MM) of colon in fetus (A,B) and newborn (C,D). The ganglia (GA) are negative (E,F). Lumen (LU) and circular muscle (CM) are marked. Lower: RT-PCR results confirming the higher level of GDNF mRNA in the colon compared to the ileum. For details see (III).



and newborn human colons (Figure 4). In contrast to a recent immunohistochemical study (Bär et al., 1997), GDNF mRNA was neither found in the neural plexuses nor in other regions of the intestine. The previous results may reflect the localization of the bound GDNF to the neurons or the distribution of other GDNF-family ligands, such as NTN crossreacting with the polyclonal antibody rather than the site of GDNF synthesis assayed by us. We found by reverse transcription-PCR that GDNF mRNA level was many folds higher in the colon than ileum (Figure 4), which is in accordance with the GDNF expression pattern found by *in situ* hybridization.

Our results by *in situ* hybridization and reverse transcription-PCR show that, in human, GDNF mRNA expression is abundant in the colon but very low in the ileum from gestational week 25 to newborn, a pattern that has not been found in the animal studies. The muscularis mucosae of rodents consists of only a few cell layers, and their entire enteric muscle layer is only slightly thicker than the human muscularis mucosae. These anatomical and scale differences between species may account for the differences in the distribution of GDNF. We cannot exclude the possibility that GDNF could have a wider distribution in earlier developmental stages, but our results suggest a major role for the muscularis mucosae, and GDNF derived from it, in the development of the innervation of the human colon at gestational week 25 and thereafter. The selective distribution of GDNF mRNA suggests critical roles for the muscularis mucosae in intestinal innervation. To our knowledge, GDNF is the first growth factor secreted by the muscularis mucosae.

By *in situ* hybridization we also found the Ret, GFRa-1 and GFRa-2 mRNA expression in the ganglionic cells of both myenteric and submucosal plexuses throughout the intestine. Interestingly, Ret and GDNF family receptors mRNAs were expressed also in the enteric segments where GDNF mRNA could not be detected. This suggests that the innervation of the gut may be affected, in addition to GDNF, by other growth factors like NTN that bind to these receptors. NTN mRNA expression in the mouse intestine has been analyzed by RT-PCR (Widenfalk et al., 1997), but at present no expression data of NTN in human or its relationship to Hirschsprung's disease has been reported.

A second signaling receptor for GDNF or NTN has been suggested based on the expression data (Suvanto, 1997) and the differences in the kidney phenotypes of GDNF and Ret –deficient mice. A second functional receptor for the GDNF family growth factors would also fit nicely with the current molecular genetic data from Hirschsprung's disease, where approximately half of the patients have no genetic explanation for their disease.

GDNF is required for ureteric budding in kidney development (IV)

In the cultures of E11 mouse urogenital blocks, the GDNF –soaked agarose beads caused supernumerous budding from the Wolffian duct. The beads distorted the branching pattern of the connecting tubules in cultures of late kidneys. Some growth factors like HGF (Davies et al., 1995) and TGF-b1 (Ritvos et al., 1995) induce the proliferation of ureteric cells and promote the elongation of the branches but these growth factors were not able to induce the budding from the Wolffian duct. The GDNF-releasing beads did

not induce budding from the Wolffian ducts of the Ret-deficient mice. Because GDNF is expressed in the nephrogenic mesenchyme, these data show that the branching of the ureter from the Wolffian duct is mesenchyme –dependent.

If the ureteric bud is recombined with heterologous mesenchymes, no branching is observed. In the recombination cultures with ureteric buds and heterologous mesenchymes supplemented with GDNF, only rat lung and kidney mesenchymes supported the branching. However, the growth or branching was not supported by salivary gland, limb bud or teeth bud mesenchymes. These data suggest that in addition to GDNF, some other mesenchymal molecules are required for the growth and branching of the ureter epithelium.

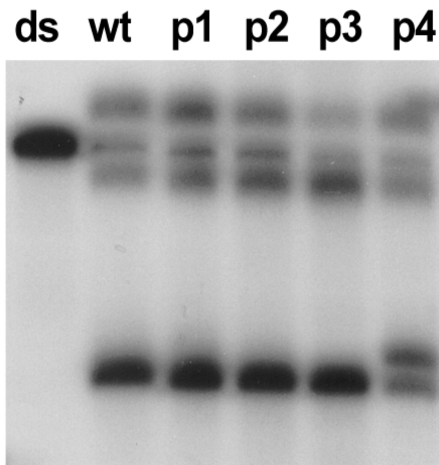


Figure 5.
Single strand conformation polymorphism (SSCP) autoradiograph showing the aberrant migration of the PCR fragment amplified from mutated p75NTR exon four in one patient (p4). Double strand (ds) and wild type (wt) controls are marked.

p75NTR in Meckel syndrome (V)

By immunohistochemistry, we found an identical distribution of immunoreactive p75NTR protein in embryonic kidneys, spleen and placenta of Meckel syndrome patients as compared to age-matched controls.

The SSCP (Figure 5) and sequence analysis revealed a heterozygous C->T substitution predicted to result in a Ser-> Leu amino acid change in the exon 4 in two Meckel syndrome patients and one carrier. We determined the prevalence of this polymorphism by minisequencing DNA pools representing the Finnish population to be approximately 10 (20/ 200) percent which was too big compared with the 1:9000 incidence of Meckel syndrome (Salonen and Norio, 1984) in Finland. No other nucleotide variations were found in the p75NTR gene in Meckel syndrome patients and the radiation hybrid panel finally located the NGFR gene outside the Meckel syndrome locus. Subsequently, the locus in Finnish Meckel syndrome patients has been refined to a less than 1 cM on chromosome 17q22 (Salonen and Paavola, 1998), and it is clear that p75NTR is not the causative gene for this syndrome.

The heterozygous polymorphism was not studied further. However, it would be interesting to study its biochemical effects, since it affects the important transmembrane region of the molecule. Based on the allele frequency, there are most likely a number of homozygous individuals for the mutation in the Finnish population. At the moment, no other inherited disease has

been mapped to the vicinity of the p75NTR gene that could be considered as a candidate disease for this developmentally interesting gene.

GDNF mutation analysis in Hirschsprung's disease and renal agenesis (unpublished)

Soon after the identification of Ret as the functional receptor for GDNF, we screened the mature protein coding region of the GDNF gene from the samples of 35 non-familial Hirschsprung's disease patients by PCR and direct sequencing. Based on the results of the importance of GDNF to the kidney development (Pichel et al., 1996) we also sequenced the samples of two families with a history of renal agenesis in two individuals from which frozen tissue was available. We did not find any alterations of the GDNF gene in these patients.

In other studies (Angrist et al., 1996; Ivanchuk et al., 1996; Salomon et al., 1996) comprising of more than 300 patients, five different variants in the entire coding region of the GDNF gene was found in seven patients. This indicates that the rate of GDNF mutations in Hirschsprung's disease is low, which is in accordance with our negative results from a relatively small number of patients.

Renal agenesis is a relatively common solitary malformation with only rare relapses in later pregnancies. It is included in a number of different syndromes, but little is known of its genetics, although there are more than 30 mouse strains with renal dysplasia or aplasia (the kidney database, 1998). Several patients with combined unilateral kidney aplasia and Hirschsprung's disease have been studied but no data of mutations in the GDNF signaling cascade has been presented thus far from patient with renal agenesis. Also our data from two patients is not sufficient to exclude GDNF gene mutations in the pathogenesis of renal aplasia.

GDNF mutation analysis in Parkinson's disease (VI)

There is strong evidence from animal experiments that GDNF protects the dopaminergic system neurons from cell death. At the moment there are no data about the levels of GDNF either in the normal or affected human brain but it would be logical to assume that the decreased levels of GDNF would result in poor survival of the dopaminergic neurons and could be a predisposing factor for Parkinson's disease. Some of the carriers of heterozygous GDNF mutations develop normally (Salomon et al., 1996) and we postulated that these individuals might be enriched within the patients of Parkinson's disease.

The coding region of GDNF gene was analyzed from 30 Parkinson's disease patients from whom we found a novel GDNF sequence variant in one patient by CFLP analysis and direct sequencing. The alteration does not change the predicted amino acid sequence or RNA folding and it was also found in one out of 20 patients without Parkinson's disease, suggesting that it represents a polymorphism in the gene rather than a disease-associated mutation. No other sequence variations were found and we concluded that GDNF mutations are not common in the sporadic form of Parkinson's disease. In our study we did not find any of the previously described GDNF mutations (Angrist et al., 1996; Ivanchuk et al., 1996; Salomon et al.,

1996) which might reflect the Finnish genetic background that somewhat differs from the rest of the world.

As described earlier, there is little evidence of a genetic contribution to most cases of Parkinson's disease (Nussbaum 1998). However, our negative mutation finding does not rule out the possible function of GDNF as an anti-Parkinson's factor. It would be interesting to analyze the GDNF protein levels and its functionality, but at present it does not seem feasible because of the low level of GDNF in the adult brain. Also the RNA analysis could be informative as Alzheimer's and Down syndrome patients were recently shown to have frame shift mutations only in the RNA level that were not detected in genomic DNA (van Leeuwen et al., 1998). Despite the great neuroprotective potency of GDNF shown in the animal experiments, the possible use of GDNF or any other neurotrophic peptide in the treatment of human patients seems distant at this point. In the animal experiments GDNF has to be adjusted parentally or even intracerebrally to avoid its degradation. In humans this kind of treatment would be difficult or impossible. Instead of using the polypeptides or vectors producing peptides, a goal in the pharmaceutical research is to create small molecules that mimic the actions of GDNF. From this point of view it would be important to know what are the intracellular signaling cascades downstream of GDNF and what are the possible ways to interfere with these mechanisms in neurodegenerative disorders, like Parkinson's disease.

CONCLUDING REMARKS

In this study, we characterized the functional receptors of GDNF and studied the biology of these neurotrophic signaling molecules in development. These and previous findings led us to the mutation analysis of GDNF and p75NTR genes as candidates for different diseases, but no disease causing mutations were found. However, we found several genetic alterations some of which could be studied further.

Our study is a part of ongoing change in genetics applied to medicine. In general, as the genetics of sporadic or multigenic diseases even more complex than Hirschsprung's disease are being studied, we need to understand the genotype-phenotype correlation better. To get to that point we need to understand the developmental function of genes and gather all the genetic information little by little in different multigenic diseases, by using the candidate gene or allelic association approach. This work will become easier with new and faster techniques such as DNA chip technology and will probably reveal rules how to interpret the consequences of a detected mutation in multigenic or sporadic diseases.

ACKNOWLEDGEMENTS

This study was carried out at the Institute of Biotechnology, University of Helsinki, and the Hospital for Children and Adolescents during 1994- 1998.

I wish to express my gratitude to the head of the Institute, Professor Mart Saarma, not only for the collaboration but also for his support and warm interest in my work. He has always been willing to help and share his time and advice in various situations.

I want to thank my supervisor, docent Hannu Sariola for his eternal optimism and vast knowledge of development and pathology and all the different aspects of scientific work. I have learned a lot from him and enjoyed our discussions about science as well as life, as we know it. Professor Juhani Rapola, my clinical supervisor, I thank for his help and advice, especially in pediatric pathology.

The Hospital of Children and Adolescents has always been very helpful. I am grateful to Professor Jaakko Perheentupa for the encouraging atmosphere for science in the clinic.

Professor Heikki Rauvala and Docent Lauri Aaltonen have given their time to pre-examine and improve the manuscript, and I am grateful for their effort.

I want to thank my numerous collaborators outside the Institute: Prof. Leena Peltonen, Dr. Paulina Paavola, Prof. Aarno Palotie, Dr. Nina Horelli-Kuitunen, Dr. Juha Rinne and Docent Risto Rintala have been important collaborators and it has been a pleasure to work with them.

The whole personnel of the Institute of Biotechnology is acknowledged for the stimulating environment as well as direct help in practical issues whenever needed. Special thanks go to the DNA synthesis and sequencing laboratory, headed by Lars Paulin.

The Developmental Biology Program of the Institute of Biotechnology led by our marvelous professor Irma Thesleff has been a good place to work. I want to thank all the members of the #development for the positive feedback and warm atmosphere. I have enjoyed the diversity of our group, both in science and social life, and I think that we make a dynamic team, also outside the sähly court.

Especially I wish to thank the members of the kidney group: Hannu, Kirsi, Anne, Marjo, Juan, Tiina, Mervi, Mika, Maxim, Illar, Jukka, Birgitta, Lilli and others for the every day life in the lab. Working with you is never boring, and maybe that is the clue to keep on going. The people in Mart's groups have also been very close to me

and Petro, Tero, Ülo, Kristiina, Maria, Urmas, Eila, Mait, Mati, Jukka, Antti, and others I thank for all the help and nice time in the former labs in Karvaamokuja and in the C-tower. Luckily, the friendship lasts longer than the shared reagents when working side by side.

Finally, I would like to thank my family and friends. My late mother was always proud of my work and I owe her a lot. I want to thank all my relatives and friends for their support, but especially my father for the discussions and advice. It has been a pleasure and a privilege to work literally under the eyes of the previous generation. However, my deepest gratitude goes to my wife Anu, not for the countless scientific discussions or help in the techniques, but for sharing the most valuable things in life with me and our daughters Alli and Linnea.

This work has been financially supported by the Foundation for Pediatric Research, The Foundation for Gastrointestinal Research, Academy of Finland, University of Helsinki, HUCH research funding, Sigrid Jusélius Foundation, and EU Biomed II.

Helsinki, beautiful May 1998

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