GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR, NEURTURIN AND THEIR RECEPTORS IN THE DEVELOPMENT, MAINTENANCE AND PLASTICITY OF THE NERVOUS SYSTEM

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ABSTRACT

Glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN) constitute the GDNF family of neurotrophic factors, which belongs to the transforming growth factor-β superfamily. The members of the GDNF family appear to play crucial roles in the development, differentiation and maintenance of various populations of vertebrate neurons (reviewed by Airaksinen et al. 1999 and Baloh et al. 2000a). Biological actions of the GDNF family members are mediated *via* a multicomponent receptor complex, which consists of glycosylphosphatidylinositol (GPI)-linked ligand-binding α–components, designated GDNF family receptor alpha (GFRα) 1 through 4, and the signal transducing receptor tyrosine kinase Ret.

The kainate-induced seizure model, a widely studied model of neuronal plasticity and human epilepsy, was applied to investigate whether the mRNAs for the GDNF receptor components Ret and GFRα1 are regulated in the adult rat brain by neuronal activity. To examine the physiological significance and ligand specificity of the GFRα proteins we generated and analyzed mice which are deficient in GFRα2. There was a special interest in investigating whether the GDNF family members, especially GDNF and NRTN, might regulate the development and/or functioning of penile erection inducing neurons in the adult rat.

The data showed that the mRNAs for the GDNF receptors Ret and GFRα1 are induced in the rat brain in response to systemic administration of kainate. The kainate induced upregulation of GFRα1 mRNA, but not Ret mRNA, was significantly suppressed in the hippocampus by the protein synthesis inhibitor cycloheximide. These results suggest that GFRα1 belongs to late-response genes, as protein synthesis is necessary for its mRNA's induction by kainate. MK-801, the N-methyl-D-aspartate (NMDA)-receptor antagonist, suppressed the behavioral seizures, but did not significantly suppress elevated levels of Ret or GFRα1 mRNA, suggesting that non-NMDA receptors are responsible for the induction of their mRNAs following kainate-induced seizures.

Analysis of GFRα2$^{-/-}$ mice showed that cholinergic innervation was almost absent in the lacrimal and salivary glands, and dramatically reduced in the myenteric plexus of the small intestine, whereas the sympathetic innervation was normal. The striking similarites in the phenotypes of mice lacking GFRα2 or NRTN, which include deficits in both parasympathetic and enteric neurons, provide strong support for the idea that GFRα2/Ret receptor complexes are the primary mediators of NRTN function *in vivo*. In addition the number of nitric oxide synthase

(NOS)-containing nerve fibers was reduced in the penis of GFRα2$^{-/-}$ mice. This points to a physiological role of GFRα2-mediated signaling in penile erection-inducing neurons.

Both NRTN and GDNF mRNAs are expressed in the shaft of the adult rat penis. In contrast to the expression of GDNF, NRTN expression was also seen in several intrapelvic organs. The GDNF family receptor components Ret and GFRα1-GFRα3 were expressed in partially distinct and overlapping populations of penile major pelvic ganglion (MPG) neurons, as well as in most other neurons of adult rat MPG. This conforms to the idea that in the adult rat certain GDNF family members may maintain not only penile parasympathetic, but also other pelvic autonomic neurons. In addition, the results showed that penile parasympathetic and sensory nerves are able to internalize and retrogradely transport to neuronal somata ^{125}I-GDNF and ^{125}I-NRTN injected into the shaft of the penis, apparently mediated by binding of these proteins to specific receptors at the axon terminals.

In conclusion, the present set of studies suggest that GDNF and its receptors Ret and GFRα1 may play a crucial role in neuronal plasticity following epileptic insults. Analysis of GFRα2-deficient mice implicates GFRα2 as a physiological NRTN receptor, crucial for the development and/or maintenance of specific postganglionic parasympathetic as well as enteric innervation. In addition, these results suggest that both NRTN and GDNF may act as a target-derived survival and/or neuritogenic factor for penile erection-inducing postganglionic neurons, as well as for other MPG neurons of the adult rat.

ABBREVIATIONS

ARTN	Artemin
AChE	Acetylcholine esterase
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
bFGF	Basic fibroblast growth factor
ChAT	Choline acetyltransferase
CREB	cAMP-responsive element binding protein
DRG	Dorsal root ganglion
E	Embryonic day
EGFR	Epidermal growth factor receptor
FG	Fluorogold
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial cell line-derived neurotrophic factor
GF	GDNF family
GFRα	GDNF family receptor alpha
GH	Growth hormone
GPI	Glycosylphosphatidylinositol
HSCR	Hirschsprung's disease
i.c.v.	Intracerebroventricularly
IGF	Insulin-like growth factor
i.m.	Intramuscularly
IR	Immunoreactive
MAPK	Mitogen-activated protein kinase
MEN 2	Multiple endocrine neoplasia type 2
MPG	Major pelvic ganglion
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NPY	Neuropeptide Y
NRTN	Neurturin
6-OHDA	6-Hydroxydopamine
PLCγ	Phospholipase Cγ
PI3-K	Phosphatidylinositol 3-kinase
PSPN	Persephin
PTC	Papillary thyroid carcinoma
RTK	Receptor tyrosine kinase
s.c.	Subcutaneously
SCG	Superior cervical ganglion
SH2	Src homology 2
TGF	Transforming growth factor
VAChT	Vesicular acetylcholine transporter
VIP	Vasoactive intestinal peptide
Y	Tyrosine residue

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications, herein referred to by their Roman numerals (I-IV):

I Reeben M*, Laurikainen A*, Hiltunen JO, Castrén E, Saarma M (1998) The messenger RNAs for both glial cell line-derived neurotrophic factor receptors, c-Ret and GDNFRα, are induced in the rat brain in response to kainate-induced excitation. Neuroscience 83: 151-159.[a]

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

III Laurikainen A, Hiltunen JO, Thomas-Crusells J, Vanhatalo S, Arumäe U, Airaksinen MS, Klinge E, Saarma M (2000) Neurturin is a neurotrophic factor for penile parasympathetic neurons in adult rat. Journal of Neurobiology 43: 198-205.[c]

IV Laurikainen A, Hiltunen JO, Vanhatalo S, Klinge E, Saarma M (2000) GDNF is expressed in penis of adult rat and retrogradely transported in penile parasympathetic and sensory nerves. Cell & Tissue Research, in press.[d]

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1. INTRODUCTION

Neurotrophic proteins were originally identified as survival factors. Ablation and transplantation studies of peripheral target tissues suggested that neuronal cell death during development is regulated by limited production of neurotrophic proteins in their projection field (Levi-Montalcini 1987). It is now known that neurotrophic factors regulate neuronal survival during development, adult nervous system plasticity, and maintenance of structural integrity (Davies 1994; Henderson 1996; McAllister et al. 1999). Since structural damage and reactive plasticity are characteristics of chronic neurodegenerative diseases, the neurotrophic factors are likely to be involved in neurodegeneration (Hefti 1994). Nerve growth factor (NGF), the first identified and most studied neurotrophic protein, was found and purified as early as 1952 from mouse sarcoma tissue based on its ability to stimulate neurite outgrowth from sensory and postganglionic sympathetic nerve cells (for review, see Levi-Montalcini 1987). For several decades NGF was the only known protein to regulate neuronal survival and differentiation. During the last two decades the field of neurotrophic factors has advanced very rapidly and a whole NGF-family of growth factors (neurotrophins) and many other neurotrophic factors have been discovered (Hefti 1997; Skaper et al. 1998).

Glial cell line-derived neurotrophic factor (GDNF) was originally isolated based on its ability to promote in culture the survival and differentiation of embryonic midbrain dopaminergic neurons (Lin et al. 1993). Later it has been shown to promote the survival of several types of neurons both in the central and peripheral nervous system (Saarma and Sariola 1999). GDNF has received attention as a potential therapeutic agent for certain neurodegenerative diseases, *e.g.* Parkinson's disease (Lapchak et al. 1997). Recently, three GDNF-releated proteins, neurturin (NRTN), artemin (ARTN), and persephin (PSPN) were identified (Kotzbauer et al. 1996; Baloh et al. 1998a; Milbrandt et al. 1998). These four neurotrophic factors comprise the GDNF family of neurotrophic factors. The GDNF family members mediate their biological actions *via* a multicomponent receptor complex, which consists of glycosylphosphatidylinositol (GPI)-linked ligand-binding receptors GDNF family receptor α:s (GFRα1 through GFRα4), and the signal transducing receptor tyrosine kinase Ret (for review, see Airaksinen et al. 1999; Baloh et al. 2000a).

The present series of studies was carried out to further elucidate the roles of the GDNF family of neurotrophic factors both in the central and peripheral nervous system. The regulation of Ret and GFRα1 mRNA in the rat brain was studied after kainate-induced seizures, a widely studied model of neuronal plasticity and human epilepsy. The GFRα2-deficient ($GFR\alpha2^{-/-}$) mice were generated and analyzed to assess the *in vivo* function of GFRα2 and the physiological relevance of its ligand specificity. Attempts

were also made to clarify whether NRTN and GDNF fulfil some of the basic criteria for being neurotrophic factors for penile erection inducing neurons. This was approached by studying the expression of their receptor components in penile parasympathetic and sensory neurons. Retrograde axonal transport of iodinated GDNF and NRTN in penile nerves was assessed to confirm the existence of functional receptors in axon terminals.

2. REVIEW OF THE LITERATURE

2.1. Introduction to the GDNF family of neurotrophic factors

GDNF and the related factors NRTN, PSPN and ARTN comprise the GDNF family, a novel family of neurotrophic factors (fig. 2.1.). The GDNF family ligands are basic, dimeric, secreted molecules which have formed a new subgroup in the transforming growth factor (TGF) -β superfamily (for reviews, see Airaksinen et al.1999; Saarma and Sariola 1999; Baloh et al. 2000a).

The first member of this family, GDNF, was discovered based on its ability to increase high-affinity dopamine uptake and promote survival and morphological differentiation of dopaminergic neurons in embryonic midbrain cultures (Lin et al. 1993). GDNF is an extracellular glycosylated disulfide-bonded homodimer that is synthesized as a 211 amino acids precursor and the mature protein consists of 134 amino acids. Interestingly, there are two potential glycosylation sites in the mature protein, which has a M_r of 18 to 22 kDa. Despite the fact that GDNF showed less than 20 percent amino acid sequence similarity with the members of the TGF-β superfamily (Lin et al. 1993), the pattern of cysteine residues made it a member of this superfamily, which is characterized by head to tail dimerization supported by a single interchain disulphide bond (Kingsley 1994). The sequence of GDNF places it also in the cysteine knot growth factor superfamily (Lin et al. 1993). The members of this superfamily are dimeric proteins characterized by a topological knot formed by three cysteines, in which two cysteines and adjacent amino acids form a covalent ring which is passed by the third cysteine (McDonald et al. 1991). Determination of the crystal structure of GDNF at 1.9 Å resolution showed that GDNF has many similar features with two structurally characterised members of the TGF-β superfamily: TGF-β2 and BMP-7 (Eigenbrot and Gerber 1997).

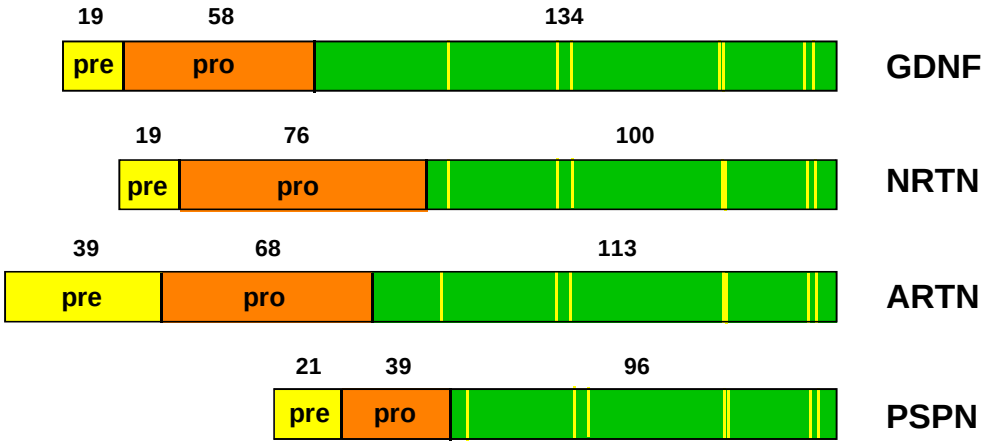

Figure 2.1. Schematic structure of the GDNF family members. The relative lengths of the mature proteins (number of amino acids), and of the pre- and proregions, as well as the relative positions of the seven conserved cysteine residues (white lines) are shown. Glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN), and persephin (PSPN). Modified from Airaksinen et al. (1999) by permission of Academic Press, copyright © 1999.

GDNF is a multipotent neurotrophic factor that has a variety of effects on cells both in the central and peripheral nervous systems, including midbrain dopaminergic neurons (Lin et al. 1993; Beck et al. 1995; Sauer et al. 1995; Tomac et al. 1995; Gash et al. 1996; Messer et al. 2000), spinal motoneurons (Henderson et al. 1994; Oppenheim et al. 1995; Yan et al. 1995), peripheral sensory and autonomic neurons (Buj-Bello et al. 1995; Ebendal et al. 1995; Oppenheim et al. 1995; Trupp et al. 1995; Forgie et al. 1999; Hashino et al. 1999), central noradrenergic (Arenas et al. 1995), as well as basal forebrain cholinergic neurons (Williams et al. 1996).

NRTN, the second member of the GDNF family, was identified on the basis of its ability to support the survival of sympathetic postganglionic neurons in culture (Kotzbauer et al. 1996). Mouse preproNRTN is a 195-amino acids long protein which has been predicted to contain 19-amino acid signal sequence and a 76-amino acid proregion. Proteolytic cleavage of the precursor should yield a 100-residue mature protein with a 42 percent similarity with mature GDNF. The mature mouse NRTN has a predicted M_r of 11.5 kDa. NRTN promotes the survival of several populations of neurons both in the central and peripheral nervous systems, *e.g.* spinal cord motoneurons (Klein et al. 1997),

sensory neurons of nodose and dorsal root ganglia (Kotzbauer et al. 1996), parasympathetic submandibular neurons (Cacalano et al. 1998), enteric neurons (Heuckeroth el al. 1998; Taraviras et al. 1999) and substantia nigra dopaminergic neurons (Horger et al. 1998; Tseng et al. 1998).

Also PSPN promotes the survival of spinal cord motoneurons and midbrain dopaminergic neurons (Milbrandt et al. 1998; Soler et al. 1999). Surprisingly, PSPN seems to be inactive on peripheral neurons. It was cloned using degenerate primers designed for the region of high similarity to GDNF and NRTN (Milbrandt et al. 1998). PSPN is about 40 percent identical with GDNF and NRTN. However, the prepro PSPN molecule is considerably smaller (156 residues) than NRTN or GDNF (195 and 211 residues, respectively) (Milbrandt et al. 1998). This may be of significance, since both GNDF and NRTN have two RXXR cleavage sites in their long prodomains, whereas PSPN has only one. Therefore, two mature species of GDNF and NRTN may exist.

ARTN, the most recently discovered member of the GDNF family, was identified by searching the high throughput genome sequences (HTGS) database, with a mature human NRTN protein sequence as a query (Baloh et al. 1998a). ARTN promotes the survival of peripheral ganglia and dopaminergic neurons *in vitro*. It is synthesized as a 220 amino acid precursor that is processed and secreted as a mature protein of 113 amino acids. Alignment of the predicted mature ARNT protein with the other GDNF family members indicates that ARTN has about 45 percent identity with NRTN and PSPN, whereas the identity with GDNF is only 36 percent. The large proregion of ARTN is separated from the mature region by multiple conserved RXXR cleavage sites. ARTN is also most probably a cysteine knot protein like the other GDNF family members (Baloh et al. 1998a). Recently Masure et al. (1999) described the molecular cloning and functional characterization of enovin, a protein which is identical with ARTN. Interestingly, they found in most tissues several differentially spliced mRNA variants for enovin, of which only two could be translated into functional enovin proteins. Some tissues seem to express only nonfunctional transcripts (Masure et al. 1999).

2.2. Introduction to the GDNF family receptors

Although the GDNF family ligands are members of the TGF-β superfamily they share a receptor system that is more similar to that of cytokines, in that a common signaling receptor is used and additional ligand-binding subunits provide the ligand specificity (fig. 2.2.) (for reviews, see Kingsley 1994; Ibáñez 1998; Airaksinen et al. 1999).

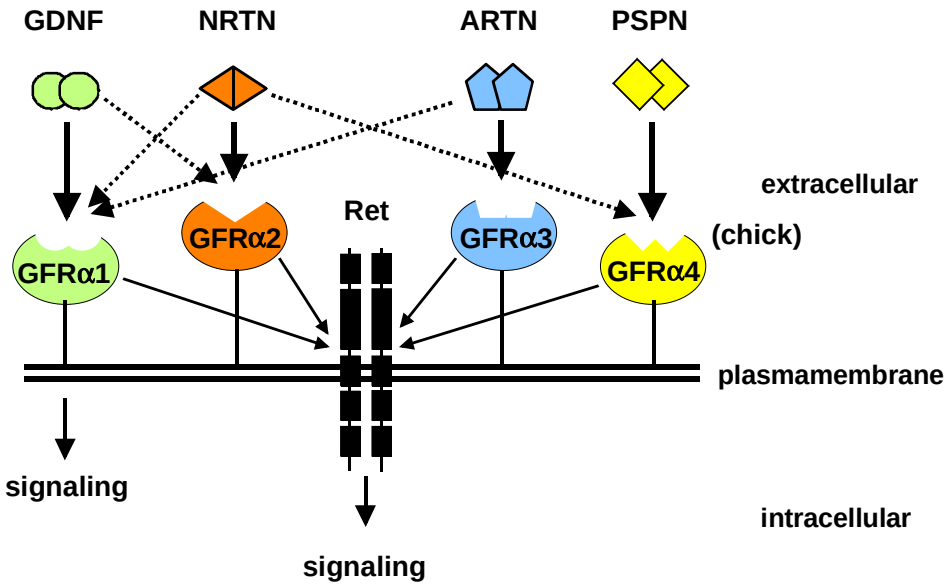

Figure 2.2. Schematic representation of GDNF family ligand-receptor interactions. All the GDNF family members activate the signaling Ret tyrosine kinase receptor *via* different glycosyl-phosphatidylinositol (GPI)-anchored coreceptors called GFRα1-4. Thick arrows represent the functional binding that activates Ret most potently, and that predominates most probably *in vivo*, whereas the interactions indicated by the dotted lines may not be physiologically significant *in vivo*. Recent experimental evidence suggests that GDNF may also signal Ret-independently *via* GFRα1. So far ligand interactions of the coreceptor GFRα4 have been characterized in the chicken only. Modified from Airaksinen et al. (1999) by permission of Academic Press, copyright © 1999.

The common signaling receptor for the GDNF family ligands is Ret receptor tyrosine kinase which is expressed in practically all cells, in which GDNF family ligands have displayed activity. The Ret gene was originally discovered as an oncogene activated by **re**arrangement during **t**ransformation (Takahashi and Cooper 1987). Germline mutations in Ret have been associated in an inherited cancer syndrome called multiple endocrine neoplasia type 2 (MEN 2), and Hirschsprung's disease (HSCR; aganglionic megacolon) (Edery et al. 1994; Hofstra et al. 1994; Romeo et al. 1994). Somatic mutations in the Ret gene have also been found in a variety of sporadic cancer types (for review, see Eng 1999). Ret was identified as the signaling receptor for GDNF by several groups almost simultaneously (Durbec et al. 1996a; Jing et al. 1996; Treanor et al. 1996; Trupp et al. 1996; Worby et al. 1996). The association of Ret and GDNF was illuminated by their complementary expression in adjacent tissues and the almost similar phenotypes of mice deficient in GDNF or Ret (Schuchardt et al. 1994; Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996; Suvanto et al. 1996).

The GDNF family ligands bind to glycosylphosphatidyl inositol (GPI)-anchored coreceptors, which are members of the GFRα family, to form a complex that subsequently activates the Ret tyrosine kinase (Jing et al. 1996; Treanor et al. 1996; Trupp et al. 1996; Baloh et al. 1997; Buj-Bello et al. 1997; Creedon et al. 1997; Klein et al. 1997; Sanicola et al. 1997). GFRα1 (formerly GDNFR-α) was the first identified member of the GFRα family, and it was discovered by expression cloning and screening for GDNF binding (Jing et al. 1996; Treanor et al. 1996). Soon after identification of GFRα1 both GFRα2 (Baloh et al. 1997; Buj-Bello et al. 1997; Jing et al. 1997; Klein et al. 1997; Sanicola et al. 1997; Suvanto et al. 1997; Widenfalk et al. 1997) and GFRα3 (Jing et al. 1997; Baloh et al. 1998b; Masure et al. 1998; Naveilhan et al. 1998; Nomoto et al. 1998; Trupp et al. 1998; Widenfalk et al. 1998; Worby et al. 1998) were cloned based on their sequence homology with GFRα1. Finally, GFRα4 was cloned by screening an embryonic chicken brain cDNA library with a GFRα1 probe at low stringency (Thompson et al. 1998).

2.3. Cross-talk within and specificity of the GDNF family ligand-GFRα system

In vitro experiments by multiple groups indicate that ligand-receptor interactions show certain specificity within the GDNF family (fig. 2.2.). Although there is some cross-talk, GFRα1 seems to be the primary coreceptor for GDNF and GFRα2 for NRTN. In cell culture both GFRα1 and GFRα2 mediate GDNF induced Ret phosphorylation with similar efficiency (Baloh et al. 1997; Jing et al. 1997; Sanicola et al. 1997; Suvanto et al. 1997). However, they seem to function in a mechanistically distinct way. *In vitro* binding experiments with soluble forms of Ret, GFRα1 and GFRα2 show that, in contrast to GFRα1, GFRα2 binds GDNF with high affinity only in the presence of Ret (Klein et al. 1997; Sanicola et al. 1997), whereas in intact cells specific interaction between GDNF and GFRα2 is also seen in the absence of Ret (Trupp et al. 1998). NRTN has a higher affinity for GFRα2 than for GFRα1 (Jing et al. 1997; Klein et al. 1997). NRTN is also more efficient in inducing Ret phosphorylation *via* GFRα2 (Baloh et al. 1997). ARTN seems to signal through Ret using GFRα3 as the primary coreceptor (Baloh et al. 1998a). Soluble GFRα3 binds directly to ARTN but not to any other GDNF family ligand (Baloh et al. 1998a). However, direct ligand-binding studies with soluble GFRα proteins have been unreliable in predicting the GDNF family interactions since Ret may modulate receptor binding specificites (Sanicola et al. 1997). In experiments where mitogen-activated protein kinase (MAPK) activity was tested in cells expressing Ret and different GFRα components, ARTN was the only GDNF family member which activated the GFRα3-Ret receptor complex (Baloh et al. 1998a). It also activated the GFRα1-Ret receptor complex suggesting that ARTN may utilize not only the GFRα3-Ret receptor complex

but also the GFRα1-Ret complex in some systems *in vivo* (Baloh et al. 1998a). PSPN seems to signal through the GFRα4-Ret receptor complex (Enokido et al. 1998). Mouse PSPN binds specifically to microtitre plates coated with chicken GFRα4-IgG as well as to human embryonic 293 kidney cells expressing chicken GFRα4 (Enokido et al. 1998). Enokido et al. (1998) provided evidence that a high concentration of NRTN can promote the survival of neurons coexpressing Ret and GFRα4, although not as effectively as much lower concentrations of PSPN. Whether the ligand specificity of the mammalian GFRα4 coreceptor is comparable to that of chicken GFRα4 remains, however, to be studied.

Recently, Baloh et al. (2000b) identified by comprehensive homologue-scanning mutagenesis two distinct sites of the GDNF molecule critical for activating the GFRα1-Ret receptor complex. They further demonstrated that chimeric mutants produced by introducing these two regions of any GFRα1 agonist (GDNF, NRTN, ARTN) into the structure of the PSPN molecule, serve as GFRα1-Ret-specific agonists *in vitro*. In addition, they reported the existence of at least one additional region critical for the alternate GFRα2-Ret interaction (Baloh et al. 2000b).

2.4. Structural similarities and differences between GFRα molecules

Rat GFRα1, the first identified member of the GFRα family is a protein made up of 468 amino acids (Jing et al. 1996; Treanor et al. 1996). Its amino acid sequence contains a signal peptide, 3 potential glycosylation sites, and 31 cysteine residues. The spacing of these cysteines is not related to that found in any of the extracellular cystein-rich domains of receptor proteins cloned before it (Jing et al. 1996). The hydrophobic cluster at the carbon terminus is preceded by a group of three small amino acids (Ala, Ser, Ser), defining a cleavage site for GPI-linkage suggesting that GFRα1 is an extracellular protein attached to cell membrane by a glycolipid anchor (Micanovic et al. 1990).

Rat GFRα2 is a 464 amino acid residue polypeptide with 6 potential glycosylation sites and it shows 49 percent identity with GFRα1 (Suvanto et al. 1997; Trupp et al. 1998). In human 30 cysteines out of 31 are present in both GFRα1 and GFRα2 with nearly identical spacing, indicating a conserved cysteine backbone structure (Baloh et al. 1997; Klein et al. 1997; Sanicola et al. 1997). Mouse GFRα3 is a 397 amino acid residue polypeptide with 32% and 37% amino acid identity with GFRα1 and GFRα2, respectively (Naveilhan et al. 1998). Like GFRα1 and GFRα2, GFRα3 contains a possible signal sequence, three potential glycosylation sites and a putative GPI-linked hydrophobic carbon terminus (Masure et al. 1998; Naveilhan et al. 1998). Analysis of the gene structure for both GFRα3 and GFRα2 confirmed that the C-terminal region, absent from GFRα3, is not the result of alternative splicing in the cDNAs (Baloh et al. 1998b). All 28 cysteines of GFRα3 were conserved in

GFRα1 and GFRα2 (Naveilhan et al. 1998). GFRα3 has a lower homology to GFRα1 or GFRα2 than GFRα1 and GFRα2 have to each other, suggesting that GFRα3 is a more distant member in this family of receptors (Masure et al. 1998; Naveilhan et al. 1998; Worby et al. 1998). GFRα3 is located in the human chromosome 5, q31.1-q31.3 region, whereas GFRα1 and GFRα2 are located at chromosome 10, region q25 and chromosome 8, region p22-p21, respectively (Masure et al. 1998).

The nucleotide sequence of chicken GFRα4 predicts a 431 amino acid protein that is more closely related to GFRα1 and GFRα2 than to GFRα3 (Thompson et al. 1998). Sequence alignment shows that GFRα4 has approximately 40% amino acid identity with both mouse and chicken GFRα1 and GFRα2, whereas it has only 27% identity with mouse GFRα3. Of the 30 cysteines that are conserved in GFRα1 and GFRα2, 28 are conserved in GFRα4 (Thompson et al. 1998). Recently a putative mouse GFRα4 gene was identified (Gunn et al. 1999) and cDNA cloned (Lindahl et al. 2000). The mouse GFRα4 protein is smaller than the other GFRα:s consisting of 263 amino acids with C-terminal sequences typical for a putative GPI-anchored protein (Lindahl et al. 2000). It shows only 53% amino acid identity with chicken GFRα4, and undergoes developmentally regulated alternative splicing in several tissues. Efficiently spliced GFRα4 transcripts have been detected in the thyroid, parathyroid, and adrenal glands that are tissues affected in MEN 2 cancer syndrome, suggesting a causal link (Lindahl et al. 2000).

Based on the sequence homology and conserved cysteine structure a putative domain structure has been suggested for the members of the GFRα family (Suvanto 1997; Airaksinen et al. 1999). There are three about 100 amino acids comprising cysteine-rich domains in the GFRα1-3 proteins, whereas the mammalian GFRα4 receptor lacks the first cysteine-rich domain characteristic of other GFRα proteins (Lindahl et al. 2000). The domains are held together by short hinge regions. The GFRα2 protein has an additional short cysteine-rich domain at the C-terminal region (Suvanto 1997).

2.5. GDNF family signaling

2.5.1. Ret-dependent activation of intracellular pathways

The structure and signal transduction pathways of the Ret tyrosine kinase receptor are summarized in fig. 2.3. The human Ret proto-oncogene, localized to chromosomal region 10q11.2, encodes the transmembrane receptor tyrosine kinase (RTK) that is mainly expressed in tissues derived from the neural crest. Ret comprises a cadherin-homology domain, a cysteine-rich region, a trans-membrane domain, and intracellular tyrosine kinase domains (Takahashi and Cooper 1987).

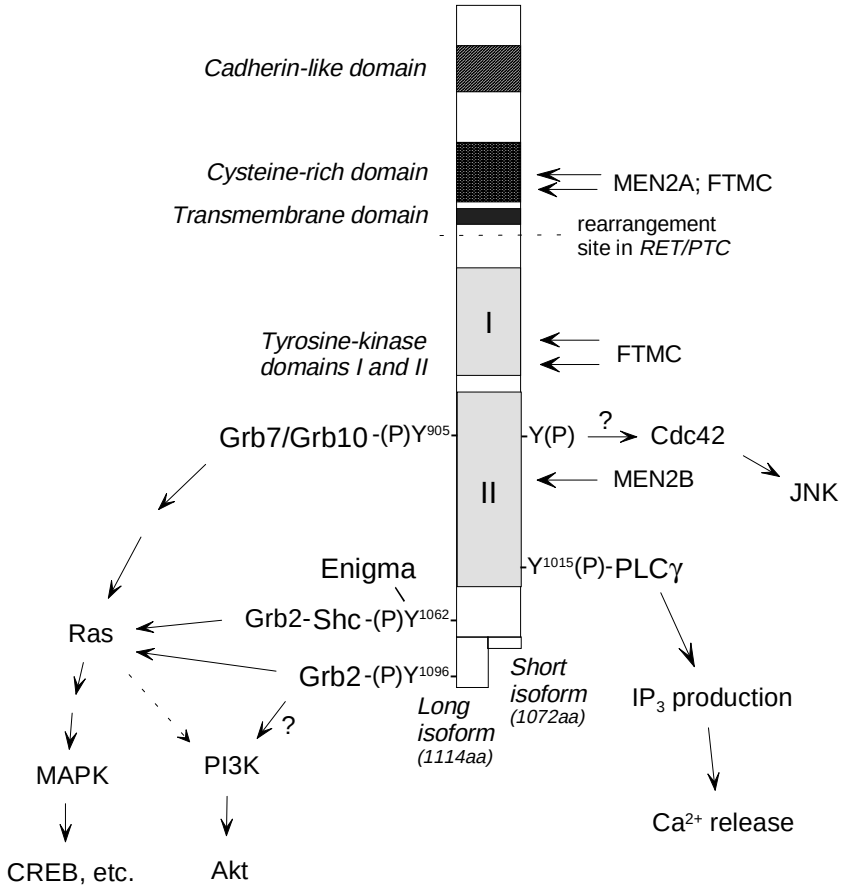

Figure 2.3. Proposed structure and intracellular signaling pathways of Ret. The phosphorylated tyrosine residues (-(P)Y) in Ret, which are used as docking sites for adaptor proteins, are shown. The figure shows that Ret activates several intracellular pathways typical of receptor tyrosine kinase signaling, including Ras-MAPK, phosphoinositol-3-kinase (PI3-K), Jun N-terminal kinase (JNK), and PLCγ–dependent pathways. The most common activating mutation sites of Ret in the cancer syndromes familial medullary thyroid cancer (FTMC), and multiple endocrine neoplasia 2 (MEN2), are shown on the right side. The dotted line shows the relative position of rearrangement sites of Ret in papillary thyroid carsinomas. The extracellular part of the Ret is not in scale. From Airaksinen et al. (1999) by permission of Academic Press, copyright © 1999.

Due to an alternative splicing the Ret protein is expressed as two major isoforms of 1072 and 1114 amino acids (Tahira et al. 1990). The intracellular domain of Ret contains 14 tyrosine residues, and the longer isoform has two extra tyrosines at the carbon terminus. Gain of function and loss of function mutations in the Ret gene lead to different syndromes, MEN 2 and HSCR, respectively (for reviews, see Pasini et al. 1996; Edery et al. 1997).

Extensive studies have recently elucidated the principles of several RTK signal transduction pathways; following

dimerization and autophosphorylation of receptors, src homology 2 (SH2) domain containing proteins are recruited to the phosphorylated tyrosine residues (Y) and activated by various mechanisms (for review, see Klesse and Parada 1999). Since Ret ligands have only recently been identified (Lin et al. 1993; Kotzbauer et al. 1996), earlier studies have used either papillary thyroid carcinoma (PTC)-Ret oncogenes (Pasini et al. 1996) or an epidermal growth factor receptor (EGFR)-Ret chimera as a source of activated Ret to study its signal transduction pathways (Santoro et al. 1994). Autophosphorylation of Ret generates docking sites for various signaling effectors; Y905 is known as a docking site for Grb7/Grb10 (Pandey et al. 1995; Pandey et al. 1996), Y1015 for phospholipase Cγ (PLCγ) (Borrello et al. 1996), while Y1062 binds both enigma and the adaptor protein Shc (Asai et al. 1996; Arighi et al. 1997; Lorenzo et al. 1997; Ohiwa et al. 1997; Durick et al. 1998; Ishiguro et al. 1999). In addition, Y1096, a tyrosine residue specific to the long isoform of Ret, is a docking site for Grb2 (Liu et al. 1996; Alberti et al. 1998). Binding of these effector proteins to specific tyrosine residues induces several signaling pathways typical of the RTK. These are phosphatidylinositol 3-kinase (PI3-K) (Santoro et al. 1994; van Weering and Bos 1997), Ras-MAPK (Worby et al. 1996; Ohiwa et al. 1997), PLCγ (Borrello et al. 1996), and c-Jun NH_2-terminal protein kinases (JNK) (Chiariello et al. 1998) dependent pathways. In addition, Ret has been recently shown to interact with Crk and Nck, which are molecules containing SH2-domains (Bocciardi et al. 1997) and to activate the cAMP-responsive element binding protein (CREB)- or Jun–mediated gene expression (Xing et al. 1998).

Naturally occurring mutations in the Ret gene have served as tools to dissect the intracellular signal pathways activated by the Ret kinase. In comparison, much less is known about exact intracellular signaling pathways triggered by the GDNF family members when they activate Ret. Both GDNF and NRTN are known to activate the MAPK signaling pathway in cultured sympathetic neurons (Kotzbauer et al. 1996). In addition, stimulation by GDNF of the human neuroepithelioma cell line SK-N-MC transfected with Ret results in PI3-K activation and PI3-K dependent formation of lamellipodia, *i.e.* cell surface protrusions that are implicated in neuritogenesis (van Weering and Bos 1997). GDNF and NRTN have also been shown to activate PI3-K in rat superior cervical ganglion neurons (Creedon et al. 1997). More recently PI3-K activation was shown to be required for GDNF induced differentiation of dopaminergic neurons (Pong et al. 1998) as well as for GDNF, NRTN and PSPN mediated motoneuron survival (Soler et al. 1999). Recently Trupp et al. (1999) reported that GDNF induces activation of MAPK and PI3-K/Akt pathways, increases CREB protein phosphorylation and c-fos expression in a motoneuron-derived cell line.

2.5.2. Mechanisms of GDNF family signaling

The traditional model of GDNF family signaling proposes quite a stringent division of labor. According to this view GFRα receptors bind ligands with high affinity, whereas the signaling receptor Ret can not bind ligands in the absence of GFRα components (for review, see Airaksinen et al. 1999). However, recently Eketjäll et al. (1999) proposed the existence of two distinct binding sites for GDNF, one formed by GFRα1 alone and another formed by a pre-associated GFRα1-Ret complex. They reported that several GDNF mutants, which failed to bind GFRα1, retained the ability to bind and activate Ret at normal levels. Although GFRα1 binding of these mutans was impaired, they still required GFRα1 for Ret activation. The data suggested that GDNF does not initiate receptor complex formation, but rather interacts with a preassembled GFRα1-Ret receptor complex. However, in contrast to this view idea Tansey et al. (2000) suggested recently that Ret and GFRα coreceptors are not preassociated *in vivo* in the absence of a ligand, but become associated after formation of the GDNF family ligand-GFRα complexes.

Surprisingly, the GFRα receptors are not always coexpressed with Ret, and they are much more widely distributed in several areas of the nervous system (Golden et al. 1998; Naveilhan et al. 1998; Kokaia et al. 1999). Various levels of GFRα1 and GFRα2 are expressed without Ret in many brain regions, *e.g.* the hippocampus and neocortex (Trupp et al. 1997; Golden et al. 1998; Yu et al. 1998; Kokaia et al. 1999). Based on the pronounced differences between the expression patterns of GFRα receptors and that of Ret, it has been suggested that there are multiple mechanisms by which Ret and the GFRα receptors can mediate the effects of the GDNF family members (Trupp et al. 1997; Yu et al. 1998). First, Ret may interact with GFRα receptors expressed in the same cells (*in cis*). Second, Ret may interact with the GFRα receptors located in the adjacent cells (*in trans*). For example, in the peripheral nervous system Schwann cells are a rich source of GDNF and GFRα1, and both are dramatically upregulated after nerve lesion (Naveilhan et al. 1997; Trupp et al. 1997). In this system GFRα1 is suggested to function *in trans* to capture and present soluble GDNF for regenerating motoneurons expressing Ret (Naveilhan et al. 1997; Trupp et al. 1997). This mode of action is further supported by the observation that GDNF can activate Ret when GFRα1 is provided in soluble form or immobilized on agarose beads (Treanor et al. 1996; Yu et al. 1998). Recently, Tansey et al. (2000) reported that activation of Ret *in cis* is a significantly more efficient mechanism for eliciting activation of signal pathways downstream of Ret and the bioactivity of Ret, as compared to *in trans* activation of Ret by the soluble GFRα1 coreceptor. Their results indicate that binding of GDNF family ligands to *cis*-expressed GFRαs and subsequent interaction

with Ret induce recruitment of the receptor complex to lipid rafts, which is of fundamental importance to GDNF family signaling (Tansey et al. 2000).

Recently two groups reported the existence of a novel signaling mechanism mediated by GFRα1 independently of Ret (Poteryaev et al. 1999; Trupp et al. 1999). This is in line with the proposed existence of two distinct binding sites for GDNF, one formed by a pre-associated GFRα1-Ret complex and another formed by GFRα1 alone (Eketjäll et al. 1999). Trupp et al. (1999) reported that GDNF promoted biochemical and biological changes in a cell line expressing high levels of GFRα1, but not Ret. GDNF treatment induced GFRα1-associated Src-like kinase activity, rapid phosphorylation of CREB and upregulation of c-fos mRNA. Poteryaev et al. (1999) demonstrated GDNF dependent phosphorylation of MAPK, CREB and PLCγ in sensory neurons of Ret-deficient mice. The exact mechanisms how GFRα1 activates the Src family are not established, but it has been suggested that a transmembrane adaptor protein links GFRα1 and Src family kinases (Trupp et al. 1999). Although there is no *in vivo* evidence, it was suggested that Ret-independent signaling may include acute plasticity-related responses, changes in gene expression and cell survival (Poteryaev et al. 1999; Trupp et al. 1999).

2.5.3. Role of other trophic factors in the signaling of the GDNF family

Recently, Krieglstein et al. (1998b) showed that GDNF requires TGF-β for exerting its full neurotrophic potential on a variety of cultured neurons including peripheral autonomic, sensory and midbrain dopaminergic neurons. Interestingly, TGF-β was not required for the GDNF-dependent survival of cultured motoneurons (Krieglstein et al. 1998b). The neurotrophic effects of GDNF were abolished by adding antibodies that neutralized endogenous TGF-β (Krieglstein et al. 1998b). The synergistic action of TGF-β and GDNF was suggested to involve the protection of GPI-linked receptors as shown by the restoration of their trophic effects after phosphatidylinositol-specific phospholipase C-mediated hydrolysis of GPI-anchored GFRα (Krieglstein et al. 1998b). The synergistic effects of GDNF and TGF-β on survival of ciliary ganglionic neurons were mediated by PI-3 kinase, since they were abolished by wortmannin which is a selective inhibitor of this enzyme (Krieglstein et al. 1998b). TGF-β has been shown to have synergistic survival-promoting effects on ciliary ganglionic neurons with the ciliary neurotrophic factor and the neurotrophins (Krieglstein et al. 1998a). More recently Schober et al. (1999) suggested that endogenous TGF-β is essential for permitting the neurotrophic effect of GDNF *in vivo*. They showed that GDNF rescues target-deprived sympathetic spinal cord

neurons but requires TGF-β as cofactor (Schober et al. 1999). The significance of TGF-β was confirmed by the fact that neutralization of endogenous TGF-β by blocking antibodies abolished the neuroprotective effect of GDNF (Schober et al. 1999). Despite the progress in the field, the mechanism of action and the physiological significance of TGF-β in GDNF-induced Ret signaling are currently unknown. The survival-promoting effect of GDNF on axotomized corticospinal neurons *in vivo* is mediated by an endogenous brain-derived neurotrophic factor (BDNF) (Giehl et al. 1998). However, the exact mechanism of a trophic cross-talk between GDNF and BDNF in the maintenance of lesioned corticospinal neurons remains unclear (Giehl et al. 1998).

2.6. Effects of the GDNF family on different neuronal populations and non-neuronal cells

The GDNF family comprises a multifunctional group of neurotrophic factors with prominent effects on many populations of neurons and nonneuronal cells. Herein only those cell populations which have received extensive experimental attention are described.

2.6.1. Midbrain dopaminergic neurons

GDNF exhibits potent survival-promoting effects on midbrain dopamine neurons both in culture (Lin et al. 1993) and *in vivo* after toxic (Hoffer et al. 1994; Sauer et al. 1995; Tomac et al. 1995; Gash et al. 1996; Choi-Lundberg et al. 1998; Rosenblad et al. 2000b) and mechanical (Beck et al. 1995) insults. Consistent with these findings GDNF is retrogradely transported to the substantia nigra after injection into the striatum (Tomac et al. 1995). The pharmacological effects of GDNF on midbrain dopaminergic neurons have been studied in several animal models both in rodents and nonhuman primates (for reviews, see Lapchak et al. 1997; Grondin et al. 1998). Due to its potent effects it is considered as a drug candidate for the treatment of Parkinson's disease. Like GDNF, also NRTN has survival-promoting effects on dopaminergic neurons *in vitro* (Horger et al. 1998), and is expressed in the developing and adult nigrostriatal system (Choi-Lundberg, Bohn, 1995; Widenfalk et al. 1997; Horger et al. 1998). *In vivo* NRTN has been shown to exert neuroprotective effects in the 6-hydroxydopamine (6-OHDA) lesion model (Horger et al. 1998; Rosenblad et al. 1999a; Rosenblad et al. 1999b). However, there seems to be important differences between the effects of GDNF and NRTN on developing and adult substantia nigra dopaminergic neurons; GDNF has wider survival, neuritogenic and hypertrophic effects as compared to the selective survival-promoting effects of NRTN (Åkerud et al. 1999; Rosenblad et al. 1999b). This may partially be explained by the observation that the effects of NRTN and

GDNF on dopamine neurons appear to be mediated by GFRα1, the preferred coreceptor for GDNF (Cacalano et al. 1998). Also the more recently discovered members of the GDNF family, PSPN and ARTN have shown some survival-promoting effects on cultured substantia nigra dopaminergic neurons (Baloh et al. 1998a; Milbrandt et al. 1998). This year, Rosenblad et al. (2000a) showed that lentiviral gene transfer of ARTN into the midbrain promoted the survival of nigral dopamine neurons after an intrastriatal 6-OHDA lesion.

2.6.2. Spinal motoneurons

GDNF increases choline acetyltransferase (ChAT) activity in embryonic motoneurons (Zurn et al. 1994) and is perhaps the most potent factor in rescuing developing motoneurons from naturally occurring or axotomy-induced cell death (Henderson et al. 1994; Oppenheim et al. 1995). It rescues adult motoneurons from avulsion-induced cell death (Li et al. 1995), attenuates the lesion-induced decrease of ChAT immunoreactivity in the nucleus of the facial nerve (Yan et al. 1995), and enhances sciatic nerve regeneration after crush injury (Naveilhan et al. 1997). It is also retrogradely transported in motoneurons (Yan et al. 1995). Recently it was reported that synapse elimination is substantially delayed in transgenic mice with muscle-specific overexpression of GNDF (Nguyen et al. 1998). In addition, GDNF is suggested to enhance transmitter release at the immature mammalian neuromuscular junction (Ribchester et al. 1998).

NRTN and PSPN also promote the survival of developing motoneurons *in vitro* (Klein et al. 1997; Milbrandt et al. 1998). In addition, PSPN rescues motoneurons from axotomy-induced cell death in the neonate rat (Milbrandt et al. 1998). NRTN, like GDNF, increases ChAT activity in postnatal motoneurons, induces neurite outgrowth in the spinal cord, and potentially protects motoneurons from chronic glutamate mediated degeneration (Bilak et al. 1999). In contrast, PSPN has no effects on mature motoneurons (Bilak et al. 1999). Recently, Soler et al. (1999) reported that GDNF, NRTN and PSPN promote the survival of chicken motoneurons *in vitro*, most probably through the PI-3 kinase pathway. They also reported that Ret, GFRα1, GFRα2, GFRα4, but not GFRα3 mRNAs are expressed in embryonic chick spinal cord (Soler et al. 1999). The expression of GFRα1 and GFRα2 receptor mRNAs in the ventral horn of the spinal cord in rat embryos had been reported earlier (Yu et al. 1998), as well as the expression of GFRα4 mRNA in the embryonic chicken spinal cord (Thompson et al. 1998). Both Ret and GFRα1 mRNAs are upregulated in spinal motoneurons after a transection or crush injury of the sciatic nerve (Naveilhan et al. 1997; Trupp et al. 1997). Further, transection upregulates GDNF and GFRα1 mRNAs in the distal nerve segment of the sciatic nerve (Trupp et al. 1997).

2.6.3. Enteric neurons

Loss of function mutations in the human Ret gene causes congenital dysgenesis of the enteric nervous system known as HSCR (Edery et al. 1994; Romeo et al. 1994). Also mice deficient in Ret lack the enteric ganglia posterior to the proximal stomach (intestinal aganglionosis) (Schuchardt et al. 1994; Durbec et al. 1996b). Targeted mutations of GDNF and GFRα1 genes in mice result in phenotypes that are remarkably similar to that of Ret-deficient mice with severe defects of enteric innervation (Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996; Cacalano et al. 1998; Enomoto et al. 1998). Also, mice lacking NRTN (Heuckeroth et al. 1999) have certain defects in the enteric nervous system although the phenotype of these mice is not as dramatic as that of the Ret-, GDNF- or GFRα1-deficient mice. These findings are consistent with the expression of Ret, GFRα1, GFRα2, GDNF and NRTN mRNAs in the developing enteric nervous system (Pachnis et al. 1993; Durbec et al. 1996b; Suvanto et al. 1996; Widenfalk et al. 1997; Golden et al. 1999).

Although genetic studies have established the critical role of Ret-mediated signaling in the development of the mammalian enteric nervous system, the mechanism of action *in vivo* is not well known. Recently, Natarajan et al. (1999) showed that multipotential enteric progenitors are capable of colonising efficiently both wild-type and Ret-deficient aganglionic bowel in organ culture. Based on these findings, they suggested that Ret functions cell-autonomously in enteric cells derived from the neural crest (Natarajan et al. 1999). Recently, Taraviras et al. (1999) reported that GDNF and NRTN promote *in vitro* survival of enteric neurons, as well as the survival, proliferation, and differentiation of multipotential enteric nervous system progenitors present in the gut of rat embryos. They also showed that a subpopulation of enteric neural crest-derived cells undergoes apoptotic cell death in the foregut of embryos lacking the Ret receptor. Consistent with these results Hearn et al. (1998) proposed that GDNF promotes proliferation of the migratory enteric neural precursor cell population once the cells have entered the gut, and is especially crucial for the differentiation of these cells into nonmigrating, nonproliferating enteric neurons. Also Heuckeroth et al. (1998) showed that GDNF and NRTN, but not PSPN, potently support the survival and proliferation of immunopurified gut neural crest precursor cells *in vitro*. The nature and age dependence of the effects of GDNF on the *in vitro* development of the precursors of enteric neurons and glia in rat has also been analysed. GDNF stimulates the proliferation of an early precursor common to enteric neurons and glia, and exerts a neurotrophic, but not a mitogenic effect on the neuronal progenitors, but does not maintain the glial progenitor cells (Chalazonitis et al. 1998).

2.6.4. Autonomic and sensory neurons

In the peripheral nervous system GDNF, NRTN and ARTN, but not PSPN, support the survival of subpopulations of sensory and autonomic neurons of rodents and chicks *in vitro* (Ebendal et al. 1995; Trupp et al. 1995; Kotzbauer et al. 1996; Moore et al. 1996; Baloh et al. 1998a; Milbrandt et al. 1998). Consistent with the wide effects of the GDNF family on the developing peripheral nervous system, Ret protein and mRNA are detected in all sensory and autonomic ganglia of developing mice (Pachnis et al. 1993; Nosrat et al. 1997; Naveilhan et al. 1998; Golden et al. 1999). Also the GFRα1, GFRα2 and GFRα3 coreceptors are expressed in sensory and autonomic ganglia throughout embryonic development (Baloh et al. 1998b; Golden et al. 1999). Recently, Lindahl et al. (2000) reported that during mouse embryonic development moderate GFRα4 mRNA expression is seen in several peripheral ganglia, including the sensory dorsal root and trigeminal as well as the superior cervical and sympathetic chain ganglia. However, due to alternative splicing of the mouse GFRα4, it is not known whether functional GFRα4 transcripts are expressed in peripheral neurons. At different stages of development, populations of peripheral neurons differ markedly in their responsiveness to GDNF and NRTN (Forgie et al. 1999). Recently, it was suggested that these differences are governed in part by the relative levels of expression of GFRα receptors (Forgie et al. 1999). Interestingly, KCl-induced depolarisation causes a marked increase in GFRα1 mRNA but a marked decrease in GFRα2 mRNA in developing sympathetic, parasympathetic, and sensory neurons (Doxakis et al. 2000). These changes are accompanied by increased responsiveness to GDNF and reduced responsiveness to NRTN. This provides evidence for a mechanism of regulation of the neurotrophic factor responses of developing peripheral neurons by neuronal activity. The physiological relevance of the *in vitro* effects of the GDNF family on the survival of the peripheral nervous system neurons is substantiated by reduction of sensory, enteric and autonomic neurons in mice having defects in the GDNF family members or their receptor components (Airaksinen et al. 1999; Rosenthal, 1999; Nishino et al. 1999).

Although genetic studies have established the critical role of various GDNF family members for the development of the peripheral nervous system, less is known about their importance for mature neurons. GDNF can prevent the death of axotomized neonatal sensory neurons (Matheson et al. 1997). Soon after birth, a population of small nonpeptidergic dorsal root ganglia (DRG) neurons switch their sensitivity from NGF to GDNF (Molliver et al. 1997). These isolectin B4 (IB4)-binding neurons remain sensitive to GNDF in adulthood. GDNF can prevent several axotomy-induced changes in these neurons including changes in GFRα1 and GFRα2 expression (Bennett et al. 1998;

Bennett et al. 2000). Recently, Leitner et al. (1999) reported that both GDNF and NRTN are retrogradely transported to the somata of adult rat DRG neurons after injection into the sciatic nerve, but not to the somata of sympathetic neurons of the superior cervical ganglion (SCG) after injection into the eye.

2.6.5. Kidney

Recent data have shown that in addition to its neurotrophic properties GDNF regulates morphogenesis of ureteric branching (for review, see Sariola and Saarma 1999). GDNF mRNA is expressed in the condensing pretubular mesenchyme that surrounds the developing ureteric system (Suvanto et al. 1996; Sainio et al. 1997). ^{125}I-labeled GDNF binds to the tips of the ureteric buds, which express mRNAs for Ret and GFRα1 (Sainio et al. 1997, Widenfalk et al. 1997). GDNF is thought to be required primarily for bud initiation from ureteric epithelium, since in organ culture it promotes dose-dependently bud formation from different parts of the Wolffian duct, and attracts ureteric branches towards the source of GDNF (Sainio et al. 1997). GDNF, combined with mechenchyme-derived soluble factors, may also induce branching morphogenesis of ureteric buds in the absence of direct contact with mesenchymal tissue (Qiao et al. 1999). Consistent with these observations GDNF increases cell motility, dissociation of cell adhesion, and migration of Ret-transfected renal epithelial MDCK cells toward a localized source of GDNF (Tang et al. 1998). Studies with GDNF-deficient mice (Pichel et al. 1996; Treanor et al. 1996) and with neutralizing antibodies to GDNF (Vega et al. 1996) are also consistent with the idea that GDNF is necessary for development of the ureteric bud. GDNF-deficient mice show regularly renal aplasia (Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996). Similarly, Ret-deficient mice show renal agenesis or severe dysgenesis (Schuchardt et al. 1994). The deficits in renal development in mice lacking GFRα1 closely resemble those found in Ret- and GDNF-deficient mice (Cacalano et al. 1998; Enomoto et al. 1998). In contrast to GDNF, which is expressed in the peripheral mesenchyme of the developing kidney, NRTN is colocalized with Ret in the developing epithelial buds (Widenfalk et al. 1997). The physiological role of NRTN in kidney morphogenesis remains to be established. However, the fact that NRTN-deficient mice do not show any defects in kidney morphogenesis suggests that the role of NRTN in kidney development may not be critical (Heuckeroth et al. 1999). Recently, Mildbrandt et al. (1998) reported that PSPN at nonphysiological concentrations was able to promote the branching of ureteric buds. These results indicate that also PSPN may possibly act as a renal ramogen and might form part of a redundant system that would explain the variability in the kidney phenotypes in GDNF-deficient mice (Milbrandt et al. 1998).

2.6.6. Testis

Recently, Meng et al. (2000) showed that GDNF, produced by Sertoli cells, regulates the fate of undifferentiated spermatogonial cells that include the stem cells for spematogenesis. They showed that mice with one GDNF-null allele suffer depletion of stem cells, whereas transgenic mice, overexpressing GDNF in spermatogonia under the influence of a testis-specific promoter, are infertile and show accumulation of undifferentiated spermatogonia (Meng et al. 2000). These results are in line with temporal and spatial distribution of GDNF, Ret and GFRα1 mRNA in the testis (Trupp et al. 1995; Suvanto et al. 1996; Widenfalk et al. 1997; Hu et al. 1999, Meng et al. 2000). GDNF mRNA expression in the postnatal testis is restricted to Sertoli cells (Trupp et al. 1995; Hu et al. 1999, Meng et al. 2000), whereas Ret and GFRα1 mRNAs are expressed in a subset of spermatogonia (Meng et al. 2000). GDNF, Ret and GFRα1 mRNAs are all expressed at their highest levels during the first postnatal week whereafter they are downregulated (Trupp et al. 1995; Meng et al. 2000).

Interestingly, NRTN and GFRα2 mRNAs show a complementary expression pattern in the testis of adult mouse (Widenfalk et al. 1997; Widenfalk et al. 2000). A NRTN mRNA signal is seen in the periphery of the seminiferous tubules, suggesting localization to the Sertoli cells, whereas GFRα2 is most probably localized in the germ cells (Widenfalk et al. 1997). However, the accurate localization of the mRNA species to different cell types in the testis of adult mice has still to be done (Widenfalk et al. 2000). NRTN overexpressing mice show a phenotype different from that of GDNF overexpressing mice (Meng et al. 2000). These mice had normal fertility and only a transient delay of spermatogenesis, indicating that the role of NRTN is not vital or that it can be substituted for.

Recently Hu et al. (1999) reported that GDNF stimulates Sertoli cell proliferation *in vitro*. This is surprising, since Sertoli cells do not express detectable levels of GDNF receptors (Meng et al. 2000). Indeed, Sertoli cell distribution is unaltered in the testes of GDNF overexpressing mice, suggesting that the proliferation of Sertoli cells is not regulated by GDNF *in vivo* (Meng et al. 2000). Nonmetastatic testicular tumors were regularly formed in older GDNF-overexpressing mice, suggesting that GDNF is also involved in the pathogenesis of germ line tumors (Meng et al. 2000).

2.7. Knockouts of GDNF family members and their receptors

Although biochemical data had provided evidence of an essential role of GPI-linked GFRα receptors in the signaling induced by the GDNF family members, the *in vivo* role of these neurotrophic factors remained controversial until characterization of the corresponding knockout mice. This

was partially due to the fact that this was the first time when a transmembrane RTK was shown to require a GPI-linked coreceptor for activation and determination of ligand specificity. The physiological roles of the GDNF family members and their receptors are highlighted by the phenotypes of their knockout mice (for reviews, see Airaksinen et al. 1999; Rosenthal 1999; Baloh et al. 2000a). Ret-deficient mice fail to form a ureteric bud and lack enteric neurons throughout the digestive tract below the stomach (Schuchardt et al. 1994). Mice lacking GFRα1 or GNDF show striking similarities with Ret-deficient mice in their phenotypes. Both die soon after birth and have severe defects in the enteric innervation and kidney development (Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996; Cacalano et al. 1998; Enomoto et al. 1998). In addition, small defects are detected in sensory, sympathetic and motor nuclei of these mice, whereas their dopaminergic neurons seem to develop normally. Despite the high similarity of phenotypes there are small differences in, *e.g.* the sympathetic ganglia. Ret-deficient mice completely lack SCG neurons in their normal location, whereas no change in the number of SCG neurons was seen in GFRα1-deficient mice, and only a 30% loss in GDNF-deficient mice (Durbec et al. 1996a; Moore et al. 1996; Cacalano et al. 1998; Enomoto et al. 1998). These similarities support the idea that Ret functions as a common signaling receptor for the GDNF family members and that GDNF acts primarily through GFRα1.

NRTN-deficient mice show defects in the parasympathetic and enteric nervous system (Heuckeroth et al. 1999). They are viable and fertile, but have a dramatically reduced parasympathetic cholinergic innervation of the lacrimal and salivary glands, and a reduced myenteric plexus innervation (Heuckeroth et al. 1999). Mice lacking GFRα3 exhibit severe defects in their SCG, whereas their other ganglia seem to be normal (Nishino et al. 1999). In the mutant embryos SCG precursor cells fail to migrate to the correct position as well as to innervate the target organs, and undergo progressive cell death after birth (Nishino et al. 1999). The subtle defects in SCG of GDNF-deficient mice (Moore et al. 1996) suggest that GDNF-GFRα3 interaction may have some physiological significance *in vivo* (Nishino et al. 1999). Similarly experiments with primary cultures of GFRα1-deficient mice nodose sensory and dopaminergic neurons have revealed that these populations have lost their responsiveness not only to GDNF but also to NRTN, suggesting interactions between NRTN and GFRα1 (Cacalano et al. 1998). However, the physiological significance of these interactions remains unclear since these neurons survive in GFRα1-deficient mice (Cacalano et al. 1998). Despite the progress made the physiological functions of ARTN, PSPN and GFRα4 still remain to be solved.

2.8. The GDNF family and activity-dependent neuronal plasticity in the brain

Several lines of evidence suggest that, in addition to their well-established survival and neurite growth promoting properties, the GDNF family members may be involved in activity-dependent neuronal plasticity in a manner similar to that of neurotrophins (for review, see Thoenen 1995). First, GDNF family ligands, as well as their receptors, are expressed in specific neuron populations of adult rodent brain, with partially overlapping patterns (Burazin and Gundlach 1999; Golden et al. 1998; Kokaia et al. 1999). Second, GDNF and NRTN, as well as their receptors are differentially regulated after various brain insults. For example, kainic acid, pilocarpine, as well as kindling-evoked seizures differentially regulate the expression of GDNF, NRTN, Ret, GFRα1 and GFRα2 mRNAs in several forebrain structures (Humpel et al. 1994; Schmidt-Kastner et al. 1994; Trupp et al. 1997; Trupp et al. 1998; Kokaia et al. 1999). Third, although the mechanisms regulating the neuronal synthesis and release of GDNF family ligands are poorly known, there is evidence suggesting that synthesis and release of GDNF can be regulated by numerous factors. Recently, Verity et al. (1998) reported that synthesis and secretion of GDNF in cells of glial and neuronal origin are differentially regulated in response to growth factors, cytokines and pharmacophores (*e.g.* fibroblast growth factors and interleukin-1β). Consistent with these observations, Krieglstein et al. (1998) showed that cholinergic stimulation regulates calcium-dependently the release of GDNF from chromaffin granules. Fourth, GDNF can regulate neurotransmitter production and release in several neuron populations, for example, in dopaminergic and serotonergic neurons (Beck et al. 1996; Pothos et al. 1998; Feng et al. 1999). It may also increase spontaneous transmitter release at the neuromuscular junction of neonatal mouse (Ribchester et al. 1998).

2.9. Neural control of penile erection

Penile erection is predominantly a nerve-mediated vascular event (for review, see Andersson and Wagner 1995). The pelvic plexus is the main source of penile erection-inducing nerve fibers, and it innervates also the internal reproductive organs, the lower urinary tract and the lower bowel (Dail 1996; Keast 1999). It is a collection of postganglionic nerve cell bodies that receive their preganglionic parasympathetic input *via* the pelvic nerves and preganglionic sympathetic input *via* the hypogastric nerves. The parasympathetic pelvic nerves are of primary importance for the smooth muscle relaxation required for penile tumescence, although the sympathetic hypogastric nerves play a role as well, the importance of which varies between mammalian species (Eckhard 1876; Langley and Anderson 1895; Sjöstrand and Klinge 1979; Dail et al. 1986; Klinge and Sjöstrand 1994). In the rat the hypogastric nerve is

preganglionic to about 20% of penile neurons in the pelvic plexus (Dail et al. 1985). In the last two decades, the rat has largely replaced the dog as an animal model to study the physiology of penile erection. One of the reasons for this is that the pelvic plexus of the rat consists of a single bilateral large ganglion, called the MPG, and small accessory ganglia, the vesicodeferential ganglia (Quinlan et al. 1989; Dail 1996; Keast 1999).

The essential role of nitric oxide (NO) as the principal neurotransmitter of the erection-inducing nerves has been well established in several mammals (Burnett et al. 1992; Klinge and Sjöstrand 1994). It diffuses into the smooth muscle cells of the penile arteries and the cavernous spaces, where it increases the synthesis of cGMP and decreases cytosolic Ca^{2+}, thus inducing relaxation (Burnett 1995). The occurrence of vasoactive intestinal peptide (VIP)–immunoreactive (IR) nerves in penile tissues has also been identified in different species (for references, see Klinge and Sjöstrand 1994; Andersson and Wagner 1995). VIP induces inconsistent and tachyphylactic relaxation of the isolated penile artery, corpus cavernosum and retractor penis muscles from several mammals (Sjöstrand et al. 1981; Willis et al. 1983). Its possible significance in the induction of penile erection is so far unsolved. In addition to VIP, several other peptides, *e.g.* neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP), have been demonstrated to occur in the nerves of penile vessels and corporal smooth muscle (Andersson and Wagner 1995). However, their roles in the physiological regulation of erection are, like that of VIP, not fully established.

Consistent with the idea that the nNOS-containing penile neurons are cholinergic, vesicular acetylcholine transporter (VAChT)- and NOS-IR terminals show coinciding profiles in the rat corpus cavernosum (Hedlund et al. 1999). The VAChT is a small carrier protein, which transports newly synthesized acetylcholine into presynaptic and prejunctional vesicles, and its immunohistochemical visualization has suggested it to be a specific marker of cholinergic nerves (Arvidsson et al. 1997). Cholinergic transmission may contribute to penile tumescence and erection at least *via* three distinct mechanisms, all of which counteract the effects of noradrenaline: First, acetylcholine may activate prejunctional muscarinic receptors on adrenergic nerve terminals and thus reduce the release of noradrenaline (Klinge and Sjöstrand 1977, 1994; Blanco et al. 1988). Second, acetylcholine may release relaxant factors from the endothelium by activation of the endothelial muscarinic receptors (Andersson and Wagner 1995). However, the variable effects of muscarinic antagonists, *e.g.* atropine, on penile erection in numerous studies with different species suggest that cholinergic muscarinic effects play no significant role in the induction of erection. In most studies atropine has been completely ineffective, whereas in some animal studies

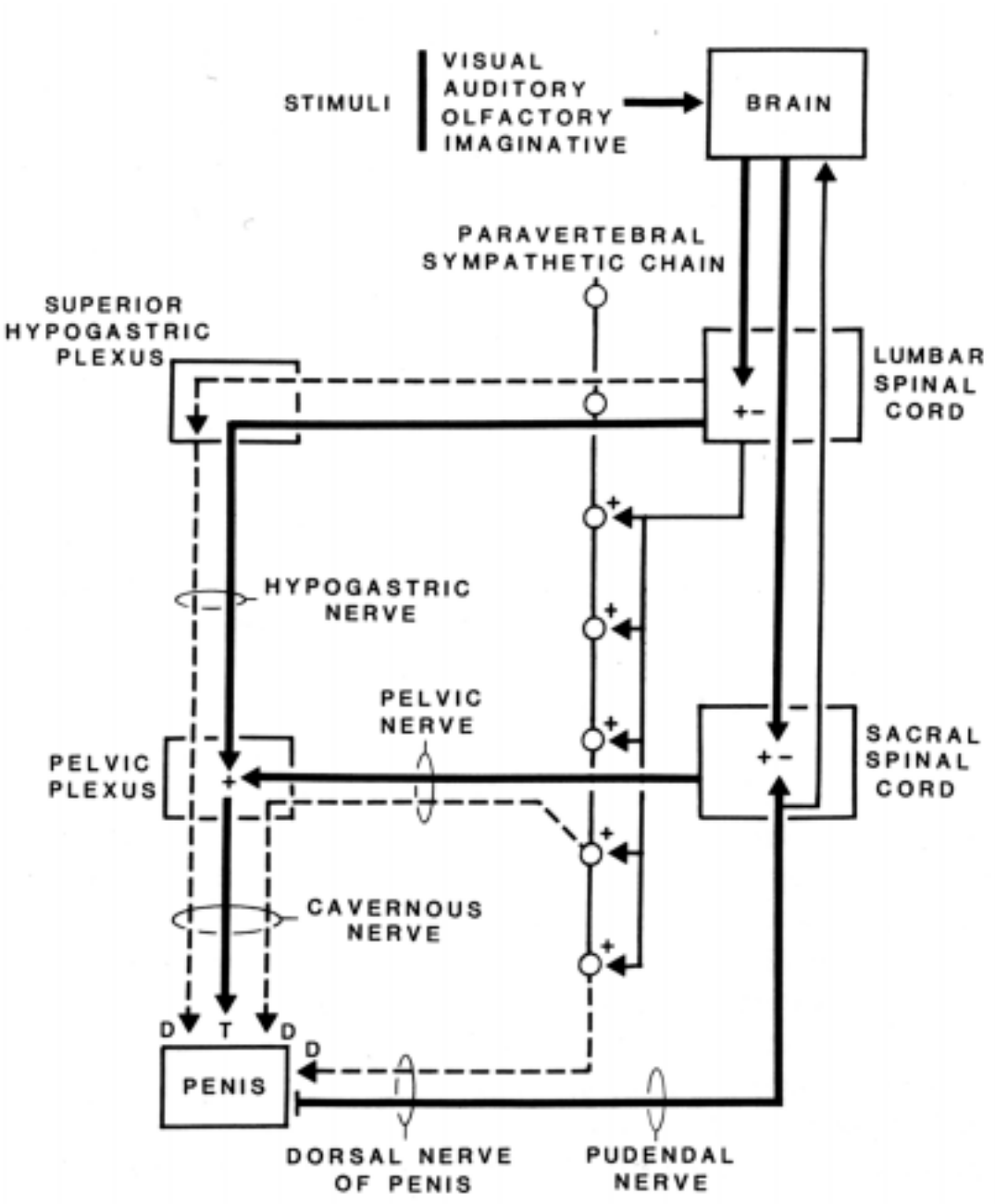

Figure 2.4. Neural control of penile erection. The diagram concisely presents the central and peripheral neural pathways involved in the control of penile erection. Penile tumescence (T) is caused by nitrergic neurons running in the cavernous nerve. Detumescence (D) is caused by noradrenergic nerves originating mainly in the paravertebral sympathetic chain ganglia (dotted lines). + indicates synaptic excitatory, and – synaptic inhibitory mechanisms. From de Groat and Steers (1988) by permission of Lippincott Williams & Wilkins, copyright © 1988 (http://www.lww.com).

atropine reduced pelvic- or cavernous nerve-evoked erections (for references, see de Groat and Booth 1993). Third, cholinergic nicotinic effects, however, play a most essential role in the induction of penile erection. Acetylcholine released from preganglionic nerve terminals, and possibly also from adjacent nerves, excites the NO releasing neurons (Klinge et al. 1988; Klinge and Sjöstrand 1994). Accordingly, ganglion-blocking drugs such as hexamethonium, almost invariably cause impotence.

Figure 2.4. summarizes the neural pathways controlling penile erection. Normal erection requires also participation of the lumbosacral somatic system which to a variable extent contracts the ischiocavernosus and bulbocavernosus muscles, and thereby contributes to the venous compression (de Groat and Booth 1993). The dorsal nerve of the penis carries along the pudendal nerve sensory fibers which form the afferent pathway of the reflexogenic erection (de Groat and Booth 1993). In contrast to psychogenic erections, which are initiated by supraspinal centers in response to various stimuli, reflexogenic erections are mediated by a sacral spinal pathway, and are preserved in patients with spinal cord injuries above the sacral segments (Bors and Comarr 1960; Comarr 1970). Psychogenic erections seem to be in part mediated *via* sympathetic pathways, *i.e.* the hypogastric nerves, since erectile function is preserved in some patients with suprasacral spinal cord lesions (Bors and Comarr 1960; Comarr 1970). Although normal erection requires participation of parasympathetic, sympathetic and lumbosacral somatic parts of the peripheral nervous system, defects in any of them do not fully exclude erectile function (Andersson and Wagner 1995).

2.10. Neurotrophic factors and the innervation of the penis

While the immediate functioning of the erection inducing neurons is relatively well characterized, very little is known about the neurotrophic factors controlling their survival and/or regeneration. In the rat model (Quinlan et al. 1989), cavernous nerve grafting can be successfully used to restore potency after surgical injury (Quinlan et al. 1991). Cavernous nerve regeneration is significantly enhanced if appropriate grafting material is combined with NGF (Burgers et al. 1991). NGF mRNA is expressed in the rat penis at levels consistent with its expression in other peripheral tissues (Te et al. 1994). Basic fibroblast growth factor (bFGF) is expressed in the penis at levels far in excess of NGF and seems to mediate the neurite outgrowth promoting activity of a penis extract in PC12 cell bioassay (Te et al. 1994). Since bFGF has both angiogenic and neurotrophic activities it may have a significant role in the physiology of erection (Te et al. 1994). Consistent with these results Tuttle et al. (1994) reported that NGF, bFGF and ciliary neurotrophic factor (CNTF) increase survival of MPG neurons cultured from female adult rat. Whether these factors increase survival of MPG neurons cultured

from male rats is not currently known. Dahiya et al. (1999) compared mRNA expression of several growth factors in penile tissues from young and old rats. TGF-β1 expression was increased, whereas expression of NGF and TGF-β3 was decreased in penile tissues of old rats. There was no change in the expression of insulin-like growth factor (IGF)-I or TGF-β2 (Dahiya et al. 1999). In penises of diabetic rats there was no significant change in the expression of IGF-I, NGF or TGF-β1 compared to normal adult rat penises (El-Sakka et al. 1999). In contrast to diabetes, neurotomy of the cavernous nerves seems to induce changes in the expression of growth factors. After unilateral cavernous nerve neurotomy IGF-I mRNA expression in rat penis showed a significant increase at three months (Jung et al. 1999). Similarly TGF-β2 expression was significantly decreased at one month but was nearly recovered at 3 months (Jung et al. 1999). NGF mRNA expression in the rat penis was not regulated by unilateral or bilateral cavernous nerve neurotomy (Jung et al. 1999).

3. AIMS OF THE STUDY

The aim of this work was to study the possible role of the GDNF receptor components, Ret and GFRα1, on neuronal plasticity following epileptic seizures and to elucidate *in vivo* functions of GFRα2. There was also an interest in investigating whether some of the GDNF family members might regulate the development and/or functioning of penile erection-inducing neurons.

The specific aims were:

- To study whether the expression of the mRNAs for the GDNF receptors Ret and GFRα1 is changed in adult rat brain in response to neuronal activity by applying the kainate-induced seizure model.

- To assess the *in vivo* importance of the coreceptor GFRα2 and its physiological ligand specificity using mice lacking a functional GFRα2 receptor.

- To investigate whether NRTN and GDNF meet some of the basic requirements for being neurotrophic factors for penile erection-inducing and penile afferent neurons.

4. MATERIALS AND METHODS

The methods used have been described in more detail in the respective communications.

4.1. Animals (I-IV)

Male adult Wistar rats (nine to twelve weeks old) were used throughout the experiments (I, III and IV). GFR$\alpha 2^{-/-}$ and wild-type mice were littermates from heterozygote mating in the hybrid 129/Sv x C57BL/6 backround (II and III). The animals were kept on a normal day and night cycle and were given free access to food and water. The experimental protocols were approved by the Committee for Animal Experiments of the Faculty of Science of the University of Helsinki.

4.2. Sample tissues (I-IV)

The tissues were treated as described in I-IV. In experiments where embryonal tissues were used (II-IV), the animals were mated overnight and the next day was regarded as embryonal day 0 E(0).

4.3. Drugs and recombinant proteins (I-IV)

The rats were anaesthetized with a s.c. injection of a combination of midazolam (0.8 mg/kg, Roche, Basel, Switzerland) and a mixture of fentanyl citrate (0.25 mg/kg) and fluanisone (8 mg/kg) (Janssen Pharmaceutica, Beerse, Belgium) (III and IV) or a combination of ketamine (105 mg/kg i.p., Parke-Davis, Solna, Sweden) and xylazin (6 mg/kg i.m., Bayer AG, Leverkusen, Germany), whereas the mice were anaesthetized with Avertin (II). Kainate (12 mg/kg s.c.) and dizocilpine maleate (MK-801, 1 mg/kg s.c.) which were both obtained from Tocris Cookson (Ballwin, MI, USA), whereas cycloheximide (8 mg/kg s.c.) and pentylenetetrazole [metrazole, 6,7,8,9-tetrahydro-5H-tetrazolo-(1,5a)azepine, 50 mg/kg s.c.] were obtained from Sigma (St. Louis, MO, USA) (I). Recombinant NRTN, GDNF and NGF were obtained from PeproTech (Rocky Hill, NJ, USA) (II and III). Rat recombinant GDNF produced by expressing the cDNA in baculovirus-infected insect cells was kindly provided by Cephalon Inc. (West Chester, PA, USA) (II and IV).

4.4. RNA preparation and Northern blot analysis (I-IV)

Total RNA was isolated from frozen samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method (I-IV) (Chomczynski and Sacchi 1987). Poly-A mRNA was purified with an Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany) (III and IV). RNA samples were fractionated on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Magna, MSI, Westborough, MA, USA). Following the transfer the membranes were fixed by UV irradiation, prehybridized, and hybridized with ^{32}P-labeled cDNA probes at 65°C in 1 M NaCl, 1% sodium dodecyl

sulfate, 10% dextrane sulfate and 100 μg/ml sonicated herring sperm DNA. After high stringency washes (0.1 x saline-sodium citrate (SSC), 0.5% sodium dodecyl sulfate at 55-63°C) the blots were exposed either to X-ray film or a Fujifilm BAS-1500 bio-imaging analyser (Fuji Photo Film, Tokyo, Japan). To compare the relative levels of mRNA in different lanes, the filter was rehybridized with a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe (II-IV).

4.5. RT-PCR and construction of probes (I-IV)

Probes were constructed by RT-PCR as described by Suvanto et al. (1996, 1997). Briefly 5 μg of total RNA purified from rat brain was incubated with random primers for 10 min at 70°C and flash frozen in liquid nitrogen. After thawing 400 units of Superscript II-reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) were added and the reaction mixture was incubated for 1 hr at 37°C. The PCR was performed in 50 μl containing 2 μl of reverse transcription mixture as a template with 100 pmol of each primer, using AmpliTag polymerase. The reaction mixture was heated at 95°C for 5 min before the enzyme was added. All reagents used in the PCR were purchased from Perkin Elmer (Roche Molecular Systems, Branchburg, NJ, USA). The products were analyzed by gel electrophoresis in 1-2% agarose gel with ethidium bromide staining. The fragments were excised from the gel and purified with Wizard PCR purification kit (Promega). After purification, the DNA fragments were ligated to pGEM-T (Promega). The identity of the cloned fragments was verified by direct sequencing with a Pharmacia A.L.F. automatic DNA sequencer. The list of the probes used in the *in situ* hybridization and Northern hybridization are presented in Table 4.1.

Table 4.1. Probes used in in situ hybridization and Northern hybridization.

Probe	Size/Vector	Nucleotides	Accession number	Used in
Rat GDNF (1,2)	388bp in pBSKII+	52-688	L15305	IV
Mouse NRTN (1,2)	587bp in pcDNA3	349-936	U78109	III,IV
Rat Ret (1)	179bp in pGEMT	21-200	U22514	III,IV
Mouse Ret (2)	3348bp in in pBSKII+	1-3348	X67812	I
Mouse Ret (1)	684bp in pGEM5Z	2534-3217	X67812	I
Rat GFRα1 (1,2)	745bp in pGEMT	294-1039	U59486	I, III
Mouse GFRα1 (2)	777bp in pT7T3D-Pac	1-777	AF012811	II
Rat GFRα2 (1)	172bp in pGEMT	85-257	AF003825	III
Mouse GFRα2 (2)	PCR fragment	1-524	NM_008115	II
Mouse GFRα2 exon 1 (1)	424bp in pT7T3	1-424	AA0478808	II
Rat GFRα3 (1)	404bp in pBSKII+	81-489	AF184920	III
Rat GAPDH (2)	812bp in pGEMT	137-949	X02231	II-IV

In situ hybridization (1)

Northern hybridization (2)

4.6. *In situ* hybridization (I-IV)

The ^{35}S-UTP-labeled sense and antisense RNA probes were prepared, and the *in situ* hybridization of sections was performed according to Wilkinson and Green (1990) with some modifications. Briefly, plasmids containing cDNAs were linearized and antisense and sense probes were generated by *in vitro* transcription. The probes were purified using Sephadex G-50 NICK columns (Amersham Pharmacia Biotech, Buckinghamshire, UK), ethanol precipitated, air-dried, and dissolved in the hybridization mixture containing 1 M dithiothreitol DTT (Promega). The probes were diluted to a final activity of 30-40 x 10^3 counts per min/µl. The paraffin sections were deparaffinized in xylene, rehydrated, fixed in 4% (wt/vol) paraformaldehyde (PFA) in phosphate buffered saline (PBS), rinsed in PBS, and treated with proteinase K (20µg/ml, Sigma) for 5-15 minutes, postfixed with 4% PFA in PBS, acetylated, dehydrated, air-dried, and hybridized with a cRNA probe overnight at 52°C. Prehybridization (1-1.5 hr at 52°C) with a hybridization mixture lacking the probe was performed. All the cryosections were treated as paraffin sections with the exception of the proteinase K treatment (1 µg/ml). After hybridization, the sections were rinsed at high stringency for 30 minutes, treated with RNase A (Boehringer Mannheim, Mannheim, Germany), dehydrated, and air dried. Sections were dipped in emulsion (Kodak NTB-2), and

exposed for 3 to 5 weeks. Subsequently the slides were developed (Kodak D19 developer), counterstained with Harris hematoxylin and mounted in Permount (Fisher Chemical, Zürich, Switzerland).

4.7. Retrograde tracing of penis-projecting neurons (III and IV)

The rats were anaesthetized with a s.c. injection of a combination of midazolam (0.8 mg/kg, Roche) and a mixture of fentanyl citrate (0.25 mg/kg) and fluanisone (8 mg/kg), whereafter 10 µl of a 4% aqueous solution of Fluoro-Gold (FG; Fluorochrome, Denver, CO, USA) was injected into the corpora cavernosa. After 10 days the rats were sacrificed under anaesthesia by transcardial perfusion with phosphate-buffered saline (PBS; 0.1 M sodium phosphate/0.14 M NaCl/2.6 mM KCl, pH 7.5) (50 ml) followed by fresh 4% paraformaldehyde (PFA; 200 ml) in PBS. MPGs and DRGs from level S1 were dissected and postfixed for 1.5-2 hours at room temperature. After fixation all samples were rinsed in PBS and processed for paraffin sectioning at 7 µm on Super Frost Plus slides (Menzel-Gläser, Braunschweig, Germany).

4.8. Iodination of proteins (III and IV)

Rat recombinant GDNF produced by expressing the gene in baculovirus-infected insect cells was radioiodinated with the Bolton & Hunter reagent (IV) (Bolton and Hunter 1973). GDNF protein (10 µg) in 20 µl of 0.1 M borate buffer, pH 8.5, was placed in dried 2mCi (1Ci=37GBq) of ^{125}I-labeled Bolton-Hunter reagent (Amersham Pharmacia Biotech), and the reaction mixture was incubated at 4ºC for 15 minutes. The reaction was stopped by adding 0.5 ml of a solution containing 0.2 M glycine in 0.1 M borate buffer, pH 8.5. Human recombinant NRTN produced in *E. coli* was radioiodinated by the lactoperoxidase method (III) (Marchalonis 1969). The following ingredients were mixed and kept at room temperature for 18 minutes in a final volume of 50 µl: 1 mCi Na^{125}I (Amersham Pharmacia Biotech), 10 µg NRTN protein, 0.5 U lactoperoxidase (Sigma), 2 µl 0.03% H_2O_2 and 0.1 M sodium phosphate buffer, pH 6.0. After the first 9 minutes of incubation, an additional 2 µl of H_2O_2 was added. The reaction was terminated by adding 3 volumes of a buffer containing 0.42 M NaCl, 0.1 M NaI and 0.1 M sodium phosphate buffer, pH 7.5, and 22 µl of 2.5% bovine serum albumin (BSA) (Sigma), in 0.1 M sodium phosphate buffer, pH 7.5. Specific activities and labeling efficiencies were calculated after trichloroacetic acid precipitation of an aliquot of the reaction mixture. Free iodine was separated and both^{125}I-GDNF and ^{125}I-NRTN were concentrated by using NanoSpin Plus columns (4000 MWCO, Gelman Sciences, Ann Arbor, MI, USA). Radioactivity was measured with a 1271 Riagamma counter (Wallac, Turku, Finland). ^{125}I-NRTN had a specific activity of 44.7 µCi/µg, and ^{125}I-GDNF had a specific activity of 61.3 µCi/µg. Labeled proteins were bioassayed for

stimulation of neurite outgrowth from embryonic day 13.5 mouse trigeminal ganglia explanted in a collagen matrix and stained with neurofilament antibodies as described below.

4.9. Immunohistochemistry (II, III and IV)

Sections were stained by standard immunohistochemical techniques. Primary antibodies were detected with an avidin-biotin-peroxidase system (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine as the chromogen (IV), or by fluorochrome conjugated secondary antibodies (II and III). All antibodies were diluted in PBS with 1% BSA and 0.1% Triton X-100. Primary antibodies used are given in Table 4.2.

Table 4.2. Primary antibodies used in immunohistochemistry.

Primary antibody	Host/Antibody type	Source/Ref.	Dilution	Used in
PGP9.5	Rabbit polyclonal	UltraClone	1:500	II
PGP9.5	Rabbit polyclonal PG9500	Affinity	1:2000	III
Substance P	Rat monoclonal NC1	Medicorp	1:300	II
ChAT	Goat polyclonal AB144P	Chemicon	1:100	II
VIP	Rabbit polyclonal VA1285	Affiniti	1:500	II
VAChT	Goat polyclonal AB1578	Chemicon	1:1000	II
TH	Rabbit polyclonal P40101-0	Pel-Freez	1:500	II, III
S100beta	Rabbit polyclonal #36	Swant	1:5000	IV
Vimentin	Mouse monoclonal RPN1102	Amersham	1:20	IV
Smooth muscle alfa actin	Mouse monoclonal #61001	Progen	1:200	IV
13AA8	Mouse monoclonal	Tienari et al. 1987	1:1	III, IV

4.10. Histochemical detection (II and III)

Intestinal samples were opened along their mesenteric border, washed in PBS, pinned on silicone without stretching, and postfixed in 4% PFA for 2 hr. The mucosal layer was removed, and the muscle layer was whole-mount stained for AChE or nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase (II). AChE histochemistry was performed as described by Tago et al. (1986), except that 1% Triton X-100 was included in the staining solution to increase penetration (II). NADPH-

diaphorase histochemistry was performed as described by Vincent (1992) (II). Briefly, whole-mount samples were incubated in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.3% Triton X-100, 0.1 mg/ml nitroblue tetrazolium (NBT) and 1.0 mg/ml NADPH at 37°C for 30-60 minutes. Following the reaction, the samples were rinsed in phosphate buffer, pH 7.4, and mounted.

Sections of the shaft of mice penis were stained for NADPH-diaphorase histochemistry as described by Carrier et al. (1995) (III). Briefly, the shafts of the penises were fixed for 5 hr in cold 4% PFA in PBS. Tissues were cryoprotected overnight in cold 30% sucrose in PBS. They were embedded in Tissue-Tek O.C.T. compound (Sakura, Zoeterwoude, The Netherlands), frozen on dry ice and stored at -70°C. Cryostat sections (10 μm) were prepared and adhered on Super Frost Plus slides. After a brief air-drying, sections were hydrated for 10 minutes with 0.1 M sodium phosphate buffer, pH 8.0. Sections were incubated with 0.1 mM NADPH, 0.2 mM NBT, 0.2% Triton X-100 in 0.1 M sodium phosphate buffer, pH 8.0, for 60 minutes at room temperature. The reaction was stopped by washing the sections in buffer, and slides were coverslipped with a Mowiol-based mounting medium.

4.11. Ganglion explant culture (II, III and IV)

E13-E13.5 mouse trigeminal ganglia were prepared and cultured in three-dimensional collagen matrix in a humidified atmosphere of 5% CO_2 in air at +37°C for 48-74 hr, fixed and stained with 13AA8 neurofilament antibodies as earlier described (Ebendal 1989; Arumäe et al. 1993). Recombinant GDNF and NRTN were applied at concentrations ranging from 1 to 100 ng/ml. For controls, trigeminal ganglia were cultured without GDNF or NRTN.

4.12. Generation and analysis of GFRα2$^{-/-}$ mice (II and III)

Generation of GFRα2$^{-/-}$ mice is described in detail in (II). Immunohistochemical as well as histochemical stainings were used to compare the innervations of GFRα2$^{-/-}$ and wild-type mice. Lacrimal gland secretion was studied by a modified Schirmer test (Humphreys-Beher et al. 1994), and the contractile activity of the gut was mesured *in vitro* with a Grass FT03 force displacement transducer coupled to a Grass model 7D polygraph. Serum growth hormone (GH) concentrations were determined by radioimmunoassay using a rat GH kit (a gift from the National Institute of Diabetes and Digestive and Kidney Diseases). Neuronal survival assays for E14 (±1) trigeminal ganglion neurons were performed as described by Davies (1995). Briefly, ganglia were digested by trypsin and cultures were purified by preplating. The cultures received GDNF, NRTN or NGF at different concentrations. After 30-35 hr the neurons were fixed and the healthy neurons were counted.

5. RESULTS

5.1. Kainate-induced expression of GFRα1 and Ret mRNAs in rat brain (I)

In normal rat brain strong GFRα1 mRNA expression was detected in the medial habenular nucleus, the reticular thalamic nucleus, and the zona incerta by *in situ* hybridization. The message was also detectable in the hippocampus in CA1-CA3 and the dentate gyrus. Lower levels of GFRα1 message were detected in the deep layers of the parietal cortex, the dorsal endopiriform nucleus, and the amygdala. In kainate-treated animals 12 hr after injection, GFRα1 mRNA was dramatically upregulated in the hippocampus, the medial habenular and reticular thalamic nuclei, and the zona incerta, as well as in the deep cortical layers and the dorsal endopiriform nucleus. In the hippocampus, a strong increase was seen in CA3 and the dentate gyrus 6 hr after kainate-treatment, with a further increase 12 hr after injection. In contrast, the levels of GFRα1 expression in CA1 and CA2 were maximal at 6 hr and were already decreased at 12 hr.

Ret mRNA was expressed at very low levels in normal rat brain. A weak signal was detected in the reticular thalamic nucleus and the zona incerta. After kainate-treatment the message was induced in the hilar region of the hippocampus, in the CA3 region and the dentate gyrus. In addition, a clear induction was seen in the medial amygdaloid nucleus and the piriform cortex.

5.2. Kainate-induced expression of GFRα1 and Ret mRNAs in the rat hippocampus (I)

Northern analysis showed that both Ret and GFRα1 mRNA levels were elevated in the rat hippocampus after kainate treatment (12 mg/kg, i.p.). The highest levels of both mRNAs in hippocampus were detected 12 hr after injection, whereas 24 hr after kainate treatment the Ret and GFRα1 mRNA levels returned almost to control levels. A maximal 2.5-fold increase in Ret mRNA and a 4-fold increase in GFRα1 mRNA were observed. Injection of the protein synthesis inhibitor cycloheximide (8 mg/kg, i.p.) 30 min prior to the kainate injection significantly reduced GFRα1 mRNA expression ($p<0.05$), but not that of Ret ($p>0.05$), whereas injection of the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 (1 mg/kg, i.p.), 30 min prior to the kainate injection, did not significantly affect the induction of Ret or GFRα1 mRNA levels.

5.3. Analysis of GFRα2-deficient mice (II)

A predicted GFRα2 null allele was created by deleting part of the first coding exon using homologous recombination in embryonic stem cells. The heterozygotes appeared normal and were fertile, and their intercrossing resulted in the birth of homozygotes at the expected frequency

(57/250), indicating no increased lethality in the homozygous animals. *In situ* analysis of GFRα2 expression in brain sections of adult wild-type and mutant mice with a GFRα2 exon 1 probe showed a strong signal in wild-type mice but no signal in mutant mice. However, similar but much weaker expression was seen in mutant mice using a full-length GFRα2 probe, suggesting the existence of a transcript under the control of the GFRα2 promoter. Consistent with *in situ* hybridization, Northern analysis with the probe spanning exons 2 and 3 showed that mutant mice lack all mRNAs, except for an upregulated transcript of about 0.5 kb. The hypothetical translation of this transcript would lead to a short, nonglycosylated, cytoplasmic peptide suggesting that mutant mice most likely represent a true null allele. At birth, the homozygous mutants appeared normal in size, but at weaning they were smaller than their wild-type littermates. Many of the homozygous mutants displayed apparent ptosis by keeping their eyes partially closed.

5.3.1. Ptosis-like phenotype and poor growth of GFRα2-deficient mice

Growth failure of GFRα2-deficient mice became visible 2-3 weeks after birth and was most prominent at the age of 4-6 weeks. At this age the difference between mutant and wild-type mice was 30-50%. Therefore, the mutant mice were easily distinguished based on size. In both sexes the difference in weight persisted beyond 4 months of age. Consistent with growth retardation, most organs were smaller in mutant mice although histological analysis did not show obvious defects in the respective organs. Possible malnutrition of mutant mice is supported by the finding that their serum albumin was reduced by 15%. The apparent ptosis was detected in many of the GFRα2-deficient mice. The ptosis was variable being unilateral in many animals, and many mice kept their eyes closed only part of the time. However, there was no difference in the sympathetic innervation of the eyelid muscles.

5.3.2. Defects in cholinergic innervation of the lacrimal and salivary glands of GFRα2-deficient mice

GFRα2-deficient mice showed almost a complete lack of immunostaining of their nerve fibers in the lacrimal gland with the PGP9.5 antibody, a panneuronal marker. The parasympathetic fibers were virtually absent, as revealed by staining for VAChT, whereas the sympathetic innervation around the blood vessels was intact. The acini were enlarged and the acinar cells appeared atrophied. In addition, preliminary analysis suggested that tear secretion was significantly reduced in mutant mice.

In addition, a dramatic reduction in the VIP-immunoreactive innervation of the sublingual and parotid salivary glands was observed. Consistent with this observation,

the number of submandibular ganglion neurons was reduced by about 80%.

5.3.3. Deficient myenteric plexus and impaired intestinal contractile activity in GFRα2-deficient mice

In the myenteric plexus of GFRα2-deficient mice, the density of the fine AChE-positive fiber network was decreased by 45%, 35%, and 15% in the duodenum, jejunum and colon, respectively. Correspondingly, substance P-positive fibers, which are known to be cholinergic, were clearly reduced in mutant mice. The density of NOS-containing nerve cell bodies and fibers in the myenteric plexus was similar between wild-type and mutant mice, as revealed by NADPH-diaphorase histochemistry. However, the density of neuronal cells in the myenteric plexus was slightly reduced, as revealed by cuprolinic blue staining. Analysis of GFRα1 and GFRα2 mRNA expression showed that GFRα1 mRNA is downregulated in the postnatal gut, whereas GFRα2 mRNA is upregulated in the bowel at postnatal day (P) 14. There were clear differences in spontaneous contractile activity of duodenum and ileum between wild-type and mutant mice. The wild-type mice showed more robust contractions and a more pronounced pattern of gut movements than the GFRα2-deficient mice. However, there was no difference in the frequency of the myogenic contractions. The contractions of the ileum, induced either by a supramaximal concentration of carbachol (3 mM) or electrical field stimulation, were clearly reduced in mutant mice. Both the carbachol- and electrical field stimulation-induced contractions were inhibited by tetrodotoxin (0.5 μM).

5.3.4. Reduced NRTN-induced neuritogenesis and cell survival of GFRα2-deficient trigeminal neurons

NRTN (5ng/ml) induced a prominent neurite outgrowth in E13 embryonic trigeminal ganglion explants of wild-type mice, whereas only few neurites were seen in the trigeminal ganglia of GFRα2-deficient mice. Interestingly, with higher concentrations (100 ng/ml), NRTN induced moderate and morphologically different neurite outgrowth also in mutant ganglia. An intermediate neurite outgrowth was induced from ganglionic explants prepared from $GFR\alpha2^{+/-}$ mice. In explants prepared from wild-type mice higher concentrations of NRTN or GDNF evoked the fasciculation of fibers.

Both NRTN and GDNF kept alive about 12% of plated E14 trigeminal neurons at nearly saturating concentrations (100 ng/ml). At lower concentrations GDNF maintained more neurons than NRTN. In contrast to wild-type neurons, mutant neurons were about ten-fold less sensitive to NRTN, whereas their response to GDNF was unaltered. Direct counting of neurons from Nissl-stained paraffin sections of trigeminal ganglia revealed no significant difference

between wild-type and GFRα2-deficient mice.

5.4. Expression of NRTN and GDNF mRNAs in the pelvic organs of the adult rat (III, IV)

In situ hybridization revealed a strong signal for GDNF in the cell layer located in the narrow area between the tunica albuginea and the cavernous tissue, whereas a weak and diffuse signal was seen in the cavernous tissue. Staining of adjacent sections with antibodies to vimentin or S100beta revealed immunoreactivity in many of these subtunical cells, whereas staining with antibodies to PGP9.5 or smooth muscle alpha actin revealed no subtunical immunoreactivity. NRTN mRNA was widely expressed in the smooth muscle of penile blood vessels and the corpus cavernosum. A signal was observed both in arteries and veins, and to some extent also in the epithelium of the urethra.

Northern analysis revealed a single NRTN transcript of 0.9 kb in all the pelvic organs studied. The highest expression was seen in the urinary bladder, penis and vasa deferentia, whereas the signal was weaker for the colon, prostate and seminal vesicles. In contrast to the wide expression of NRTN, a GDNF transcript of 4.4 kb was detected only in the shaft of the penis, while there was no detectable signal in the urinary bladder, colon, prostate, vasa deferentia or seminal vesicles.

5.5. Expression of NRTN and GDNF mRNAs in the penis after cavernous nerve axotomy (IV)

Expression of GDNF mRNA in the shaft of adult rat penises was clearly lowered 1, 3 and 7 days after bilateral cavernous nerve axotomy, and had almost regained the level of the sham operated rats on day 14. The level of NRTN mRNA transcripts was the same in the sham operated and bilateral neurotomy group on days 3 and 14, and only slightly, if at all, lowered on days 1 and 7.

5.6. Expression of Ret and GFRα1-3 mRNAs in the MPG and the S1 DRG of the adult rat (III, IV)

Ret and GFRα2 mRNAs were expressed in most if not all neurons of the adult rat MPG, whereas GFRα1 and GFRα3 mRNAs were expressed only in subpopulations of the MPG neurons. Both GFRα1 and GFRα3 coreceptors were expressed in virtually all big-sized, *i.e.* sympathetic neurons but only in subpopulations of the small-sized, *i.e.* cholinergic neurons of the MPG. Accordingly, the expression of Ret and GFRα2 mRNAs was seen in virtually all penis-projecting, *i.e.* fluorogold (FG)-positive MPG neurons, while the expression of GFRα1 and GFRα3 mRNAs was seen in about 54% and 5% of the penis-projecting neurons, respectively.

Quantitative data of the expression of Ret, GFRα1, GFRα2 and GFRα3 mRNAs in

penile MPG and S1 DRG neurons of adult rat are presented in Table 5.1. There was a differential distribution of receptor mRNAs within the S1 DRG cells according to cell size. Ret, GFRα2 and GFRα3 mRNAs were expressed preferentially in small and intermediate-sized cells, whereas GFRα1 mRNA was expressed preferentially in intermediate- and large-sized cells. There was no detectable expression of NRTN or GDNF mRNAs in the MPG or the S1 DRG of adult rat.

Table 5.1. Quantative data of the expression of Ret and GFRα1-3 mRNAs in penile MPG and S1 DRG neurons.

	MPG (%)	S1 DRG (%)
Ret	100.0 (384)	62.0±3.5 (398)
GFRα1	53.9±2.5 (396)	57.1±4.0 (443)
GFRα2	98.4±1.5 (426)	6.3±2.4 (459)
GFRα3	5.3±2.6 (414)	45.6±6.3 (505)

The expression of receptor mRNAs in FG-labeled neurons was quantified unilaterally in three rats. Values represent mean±S.D.; total number of neurons analyzed in parentheses.

5.7. Retrograde axonal transport of ^{125}I-NRTN and ^{125}I-GDNF in penis-projecting neurons (III, IV)

Both ^{125}I-NRTN and ^{125}I-GDNF induced strong neurite outgrowth in mouse embryonic E13 trigeminal ganglia, while there were no neurites in control ganglia cultured in the absence of neurotrophic factors. ^{125}I-labeled and nonlabeled proteins exhibited similar neuritogenic efficacy at all concentrations tested (1-20 ng/ml). 24 hrs after injection of 200 ng of ^{125}I-NRTN or ^{125}I-GDNF into both corpora cavernosa, autoradiography revealed clustered silver grains in the MPG and the S1 DRG. The retrograde transport was blocked when ^{125}I-proteins were injected together with excess unlabeled proteins, whereas excess cytochrome c did not block the transport.

5.8. Loss of NADPH diaphorase staining in the penile nerves of GFRα2$^{-/-}$ mice (III)

On histochemical examination of the proximal part of the shaft and the crura penis from wild-type and GFRα2$^{-/-}$ mice, a significantly different pattern of NADPH diaphorase-positive nerve fibers was noted. GFRα2$^{-/-}$ mice had very few NADPH diaphorase-positive fibers in the dorsal penile nerves, while the fiber density in wild-type mice was similar to that reported in rats (Carrier et al. 1995). There was no clear difference in the staining pattern of nerves supplying cavernosal vessels in the crura. However, in the crura the number of NADPH diaphorase-positive fibers was significantly reduced outside the perivascular area. In contrast, no difference was noted in the density of the tyrosine hydroxylase-positive fibers in the crura penis.

6. DISCUSSION

6.1. Regulation of GFRα1 and Ret mRNAs in rat brain by kainate-induced seizures (I)

Kainate-induced seizures, a widely studied model of neuronal plasticity and human epilepsy (Ben-Ari 1985), have been shown to increase in rat hippocampus gene expression of several trophic factors, including GDNF (Humpel et al. 1994). The results of the present study show that GFRα1 and Ret mRNAs are differentially expressed and upregulated in adult rat brain following kainate-induced seizures. In agreement with the results of other studies (Trupp et al. 1997; Widenfalk et al. 1997; Kokaia et al. 1999), GFRα1 mRNA expression was widespread as compared to Ret mRNA, which was expressed at very low levels. Ret and GFRα1 mRNAs were upregulated in several brain structures, with maximum levels in the hippocampus 12 hr after kainate administration. Ret mRNA upregulation was detectable in the hippocampal formation, reticular thalamic nuclei, zona incerta, amygdala and piriform cortex, whereas GFRα1 mRNA was upregulated in the habenular and reticular thalamic nuclei, hippocampus, deeper layers of the parietal cortex and the dorsal endopiriform nucleus. Recently Trupp et al. (1998) reported that in rat brain also the GFRα2 mRNA levels could be induced by kainic acid treatment (0.35μg/0.5μl, i.c.v., bilaterally). A pronounced elevation was seen in thalamus and the dentate gyrus 12 hr after stimulation with kainic acid (Trupp et al. 1998). GDNF mRNA expression is increased in scattered neurons in the dentate granule layer 3 hr after injection of kainate (12 mg/kg i.p.), and after 6 hr almost all dentate granule cells expressed GDNF mRNA (Humpel et al. 1994). Trupp et al. (1997) reported that 12 hr after kainic acid treatment (0.35μg/0.5μl, i.c.v., bilaterally), increased GDNF mRNA levels were observed in all regions of the hippocampus. Thus, there are similarities and differences in the spatial and temporal expression patterns of GDNF, Ret and GFRα1 mRNAs in rat brain before and after kainate-induced epileptic seizures. The expression patterns of GFRα1 and Ret are in agreement with the neuroprotective properties of GDNF. Martin et al. (1995) showed that GDNF prevents kainate-induced seizures and seizure-associated neuronal cell loss in the hippocampus, thalamus and amygdala. GDNF provides also potent protection against brain infarction (Wang et al. 1997) and transient forebrain ischemia (Miyazaki et al. 1999).

While the present study (I) was being reviewed, Trupp et al. (1997) using *in situ* hybridization reported in detail GDNF receptor mRNA expression in normal rat brain, as well as following kainate-induced seizures. Although they applied i.c.v. injection of kainate their data essentially agree with our results. These results are also well in line with upregulation of Ret and GFRα1 mRNAs in rat hippocampus after

kindling-evoked seizures (Kokaia et al. 1999).

There are at least two possible mechanisms for the regulation of Ret and GFRα1 gene expression after kainate-induced seizures. First, their transcription could be activated directly by depolarizing neuronal activity, glutamate release and calcium influx. These mechanisms have been suggested to explain the upregulation of GDNF mRNA after seizures (Humpel et al. 1994; Ho et al. 1995), as well as of neurotrophins (NGF and BDNF) (Zafra et al. 1990; Gall et al. 1991), and numerous other genes induced in the hippocampus by kainate (Nedivi et al. 1993; Kasof et al. 1995). Second, it is possible that upregulation of these receptors may be mediated by GDNF. This idea is consistent with the fact that GDNF upregulation occurs earlier than that of Ret and GFRα1. For several reasons the data presented suggest that upregulation of GDNF-receptors is primarily due to the neuronal activity *per se* and not to the neuronal cell death occurring after kainate-induced seizures. First, MK-801 blocks kainate-induced behavioral seizures and cell death (Humpel et al. 1994), but does not block kainate-induced upregulation of Ret and GFRα1 mRNAs. Second, although pentylenetetrazole-induced seizures do not produce neuronal cell death (Schreiber et al. 1993) they elevated the levels of GDNF receptor mRNAs.

A co-expression of trophic factors and their receptors within the hippocampal formation is well documented for neurotrophins. Many hippocampal cells express trkB and trkC mRNAs (Merlio et al. 1993), as well as their ligands, BDNF and neurotrophin (NT)-3 (Ernfors et al. 1990), while trkA, the cognate receptor of NGF, is not expressed at all in the hippocampus. In comparison with the BDNF and NGF, the time-course of the induction of GDNF and its receptors seems to be slower (Zafra et al. 1990; Trupp et al. 1997). The present study shows that, following an epileptic insult, Ret and GFRα1 mRNAs are upregulated in several brain regions. However, the level and localization of mRNA do not necessarily reflect the level and localization of proteins. For example, BDNF mRNA is upregulated in rat hippocampus more than 20-fold by kainate-induced seizures, whereas the BDNF protein is elevated only two- to three-fold (Lindvall et al. 1994). Recently, Mikuni et al. (1999) reported that the GDNF protein level was upregulated in dentate granule cells 3 hr after kainate-induces seizures, continued to increase until post-injection day 4, and returned to the control level by day 7. It has not been reported whether also the protein levels of Ret and GFRα1 are elevated after kainate-induced seizures.

Consistent with the results of several groups (Trupp et al. 1997; Golden et al. 1998; Yu et al. 1998), GFRα1 mRNA was seen without Ret mRNA in several brain regions, most notably in the cortex. In these brain regions GFRα1 could capture and present GDNF or NRTN to afferent axons expressing Ret (Trupp et al. 1997; Yu et al. 1998). This interaction ”*in trans*” has been suggested to

occur, *e.g.* between thalamic afferent neurons that transcribe high levels of Ret, and cortical neurons, that show high expression of GFRα2 mRNA (Yu et al. 1998). Alternatively, GFRα1 could signal by itself, as has been previously reported (Poteryaev et al. 1999; Trupp et al. 1999). It is also possible that other signaling components may exist in addition to Ret. This idea is supported by the observation that GDNF promotes the survival of postnatal rat cochlear neurons, which express GFRα1 mRNA but lack detectable levels of Ret mRNA (Ylikoski et al. 1998).

In conclusion, the present results suggest that increased levels of the GDNF receptors, Ret and GFRα1, may further amplify the putative responses to GDNF following epileptic seizures. This insult–induced cascade may provide and facilitate a local trophic support within the hippocampus that both protects against neuronal death following an epileptic insult and maintains or reorganizes the neuronal connections in the normal adult hippocampus.

6.2. Physiological role of the coreceptor GFRα2 (II)

6.2.1. Physiological pairing of GFRα2 and neurturin

Results from *in vitro* assays suggest that GFRα2 mediates activation of Ret by both NRTN and GDNF (Baloh et al. 1997; Buj-Bello et al. 1997; Creedon et al. 1997; Klein et al. 1997). However, the ligand specificities of the GFRα receptors appear complex, differing in the presence and absence of Ret. Transcripts of Ret and the GFRα receptors are expressed in temporally and spatially distinct, but partially overlapping patterns in the developing embryo and adult mice (Golden et al. 1998; Naveilhan et al. 1998; Golden et al. 1999). NRTN and GFRα2 show complementary expression patterns in several organs, *e.g.* developing teeth and salivary gland, suggesting that NRTN and GFRα2 may be physiologically coupled *in vivo* (Luukko et al. 1997; Widenfalk et al. 1997; Golden et al. 1999). To assess the *in vivo* function of the coreceptor GFRα2 and the physiological ligand specificity, GFRα2$^{-/-}$ mice were generated. The striking similarity of the phenotypes of mice lacking GFRα2 or NRTN, which includes deficits in parasympathetic and enteric neurons, support the idea that GFRα2 serves as a primary physiological coreceptor for NRTN (Heuckeroth et al. 1999). Both GFRα2$^{-/-}$ and NRTN$^{-/-}$ mice suffer a nearly complete lack of parasympathetic innervation of the lacrimal and salivary glands, show comparable reduction in the nerve fiber density of the myenteric plexus and disordered gastrointestinal motility. No striking abnormalities were observed in the central nervous system of GFRα2$^{-/-}$ mice despite the fact that adult mouse brain express high levels of GFRα2 (Golden et al. 1998). The lack of severe defects in the central nervous system may reflect the

tendency of its neurons to rely on multiple trophic factors simultaneously (Snider 1994).

There was no significant difference in the number of trigeminal ganglion neurons between wild-type and GFRα2$^{-/-}$ mice. Moreover, in GFRα2$^{-/-}$ mice the nodose ganglia and the DRGs appeared to be of equivalent size as compared to those in wild-type mice. In contrast, NRTN$^{-/-}$ mice have been shown to display a 70% reduction in the number of GFRα2-expressing trigeminal ganglion neurons, and a 45% reduction in GFRα2-expressing DRG neurons (Heuckeroth et al. 1999). These defects, which are small in the absolute number of neurons, were not detected in GFRα2-deficient mice, probably due to differences in detection methods. Despite the striking similarity between the GFRα2$^{-/-}$ and NRTN$^{-/-}$ mice, they display one clear difference. GFRα2$^{-/-}$ mice grow poorly after weaning, whereas NRTN$^{-/-}$ mice grow normally (Heuckeroth et al. 1999). The etiology of the growth difference remains unclear. One possibility is that GDNF could substitute NRTN and activate GFRα2 in areas important for growth (Heuckeroth et al. 1999). Another possibility is that the difference in growth reflects differences in strain genetic background or diet.

The idea of physiological pairing of NRTN and GFRα2 is further enforced by the observation that NRTN induced a prominent neurite outgrowth in E13 embryonic trigeminal ganglion explants of wild-type mice, whereas only a few neurites were seen in the trigeminal ganglia of GFRα2-deficient mice. Moreover, GDNF induced neurite outgrowth in trigeminal ganglia explants of GFRα2-deficient and wild-type mice appeared indistinguishable. Interestingly, with higher concentrations, NRTN induced moderate neurite outgrowth also from mutant ganglia. In dissociated neuronal culture GFRα2$^{-/-}$ sensory neurons no longer responded to physiological concentrations of NRTN. These data are consistent with previous *in vitro* results which show that GFRα2 is the primary coreceptor for NRTN, but not for GDNF, and that NRTN may at high concentrations signal *via* GFRα1 (Baloh et al. 1997; Buj-Bello et al. 1997; Jing et al. 1997; Klein et al. 1997). The phenotypes of GDNF-family ligand- and receptor-deficient mice are summarized in Appendix I.

6.2.2. Role of GFRα2 in development/ maintenance of enteric neurons

GFRα2-deficient mice have a reduction in the density of the nerve fibers of the myenteric plexus and disordered gastrointestinal motility, whereas the reduction in number of enteric neurons is minimal. These defects are strikingly similar to those observed in NRTN$^{-/-}$ mice (Heuckeroth et al. 1999). In culture both GDNF and NRTN similarly increase proliferation and process formation of developing enteric neurons (Chalazonitis et al. 1998; Hearn et al. 1998; Heuckeroth et al. 1998). Although the effects of these factors are indistinguishable in culture, their

physiological role *in vivo* seems to be very different. GDNF- as well as GFRα1-deficient mice fail to develop their enteric nervous system, most probably as a result of insufficient proliferation of enteric precursor cells and increased cell death (Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996; Cacalano et al. 1998; Enomoto et al. 1998), whereas the defects in the enteric nervous system in NRTN- and GFRα2-deficient mice are much smaller without significant reduction in the number of enteric neurons. These results suggest that GDNF and GFRα1 are required for early development of the enteric nervous system, whereas NRTN and GFRα2 are crucial for postmitotic myenteric neurons. This idea is further supported by the observation that GFRα1 mRNA is downregulated in the postnatal gut of wild-type mice, whereas GFRα2 mRNA is upregulated in the bowel at postnatal day 14. Interestingly, GFRα2 mRNA expression is variable along the gastrointestinal tract being most prominent in the small bowel. The expression of GFRα2 mRNA is increased at the time when the development of the cholinergic myenteric plexus is accomplished (Vannucchi and Faussone-Pellegrini 1996). Moreover, expression of GDNF in the postnatal gut is very low, whereas NRTN expression is increased to a high level in the adult gut (Golden et al. 1999).

6.2.3. Role of GFRα2 in development/ maintenance of parasympathetic neurons

The parasympathetic innervation was almost absent in the lacrimal and salivary glands of GFRα2-deficient mice. This is consistent with the expression of GFRα2 and Ret mRNAs in parasympathetic ganglia within the submandibular gland as well as in the pterygopalatine ganglion, and with the high level of NRTN mRNA expression in the submandibular and lacrimal glands of developing mouse (Golden et al. 1999). Interestingly, not all parasympathetic neurons were lost, as the ciliary ganglion and the pupillary reflex of GFRα2-deficient mice appeared normal. However, adult NRTN-deficient mice have almost a 50% reduction in the number of ciliary ganglion neurons (Heuckeroth et al. 1999). The size of individual otic ganglion cells, but not the number of neurons, was reduced in neonatal NRTN-deficient mice as compared to wild-type animals (Heuckeroth et al. 1999). Whether GFRα2-deficient mice have a similar defect in the ciliary and otic ganglia remains to be studied. The observation that GFRα2-mediated signaling is important for the development or maintenance of the parasympathetic neurons is consistent with the defect in parasympathetic neurons in mice lacking Phox2a or Phox2b, which are transcription factors that regulate the expression of Ret (Morin et al. 1997; Lo et al. 1998; Pattyn et al. 1999). Although GFRα2 is widely expressed in the developing as well as

in the mature peripheral nervous system (Baloh et al. 1997; Naveilhan et al. 1998; Golden et al. 1999; Nishino et al. 1999), GFRα2$^{-/-}$ mice show most prominent defects in their parasympathetic ganglia. The lack of major abnormalities in sympathetic ganglia, *e.g.* the superior cervical ganglion or sensory ganglia, is likely attributable to a functional redundancy among GFRα proteins.

6.3. GDNF family ligands and their receptors in the innervation of the penis (III, IV)

6.3.1. Expression of GDNF and NRTN mRNAs in the penis and other pelvic organs

The discovery of GDNF and NRTN was soon followed by the demonstration of their ability to promote survival of several neuronal populations in the peripheral and central nervous system (Airaksinen et al. 1999; Saarma and Sariola 1999). However, the possible significance of GDNF family ligands as neurotrophic factors for pelvic neurons has not been previously studied. In this study it is shown that both NRTN and GDNF mRNAs are expressed in the shaft of adult rat penis. *In situ* hybridization revealed differences between the expression patterns of NRTN and GDNF in the adult rat penis. NRTN mRNA was expressed in penile smooth muscle in close coincidence with the neuron-target contact, whereas GDNF expression was focused to the subtunical cell layer in the shaft of the penis. Our results suggest that the GDNF mRNA expressing cells are likely to be of mesenchymal origin, since many subtunical cells displayed immunoreactivity for vimentin, which is considered as a marker of mesenchymal cells (Leader et al. 1987). In contrast to the expression of GDNF, NRTN expression was also seen in several intrapelvic organs. These results suggest that endogenously produced NRTN may work as a target-derived neurotrophic factor in several pelvic organs, whereas the role of GDNF may be more restricted. However, the possibility that GDNF mRNA is expressed in some pelvic organs in amounts below the level of detection can not be exclude. This could be expected since GDNF receptor components are widely expressed in the MPG neurons of adult rat.

In order to find out whether GDNF and NRTN mRNA synthesis in the penis is under neuronal control, expression of their mRNAs was studied after bilateral cavernous nerve axotomy. The observation that NRTN mRNA expression in the shaft of the penis was not remarkably altered after bilateral cavernous nerve axotomy is consistent with the results of several denervation experiments of autonomic nerves in neonatal and adult animals. For example, NGF mRNA levels remain unchanged after sympathectomy in the neonatal rat heart as well as in the iris of adult rat (Shelton and Reichardt 1986; Clegg et al. 1989). Surprisingly, GDNF mRNA expression was transiently downregulated in the penis following bilateral cavernous nerve axotomy. The cellular and molecular

mechanism of this phenomenon is, however, still unknown, and its physiological significance remains to be established.

6.3.2. Expression of Ret and GFRα1-3 mRNAs in the MPG and the S1 DRG of the adult rat

The results also demonstrate that practically all the penis-innervating as well as most other MPG neurons express mRNAs for the NRTN receptor components GFRα2 and Ret. In addition, the coreceptors GFRα1 and GFRα3 were detected in subpopulations of penile MPG neurons as well as in most other MPG neurons. These data conform to the idea that in adult rat certain GDNF-family members may be maintenance factors not only for pelvic parasympathetic, but also for pelvic sympathetic neurons. The wide coexpression of GFRα1, GFRα2 and GFRα3 mRNAs in MPG neurons, escpecially in sympathetic MPG neurons, suggests that several GDNF family ligands may exert trophic effects on the same neurons in adult rat MPG. Similar partially overlapping expression of GFRαs has been demonstrated in the central nervous system as well as in other parts of the peripheral nervous system (Golden et al. 1998; Naveilhan et al. 1998; Golden et al. 1999). Interestingly, a subpopulation of small-sized neurons in the MPG express mRNA only for the coreceptor GFRα2, but not for GFRα1 or GFRα3. Whether there is a functional distinction between neurons expressing mRNAs for different combinations of GFRαs remains to be studied.

6.3.3. Retrograde axonal transport of ^{125}I-GDNF and ^{125}I-NRTN in penis projecting MPG and DRG neurons

In addition to expression in the target cells, neurotrophic factors need to be internalized and retrogradely transported in order to be able to exert their biological effects in neuronal somata (Korsching 1993). Indeed, retrograde axonal transport from nerve terminals to neuronal somata is an important criterion for the physiological significance of a neurotrophic factor candidate. Recently Leitner et al. (1999) reported selective receptor-mediated transport of NRTN and GDNF in sensory and motoneurons of adult rat. After intrastriatal injection GDNF is retrogradely transported also by mesencephalic dopamine neurons (Tomac et al. 1995). The present study demonstrates the binding, uptake and retrograde axonal transport of ^{125}I-NRTN and ^{125}I-GDNF in penis-projecting MPG and DRG neurons of adult rat. This supports the hypothesis that endogenously produced NRTN and GDNF may have physiological roles as trophic factors for these neurons. The fact that ^{125}I-labelled GDNF family ligands, with a neurite outgrowth promoting activity comparable to that of the unlabeled parent proteins, were transported in the presence of a 100-fold excess of cytochrome c but not in the presence of a similar excess of parent proteins, suggests that the retrograde

transport was mediated by binding to specific receptors on the axon terminals. This is further supported by the observation that, 24 hr after injection of ^{125}I-labelled proteins into the shaft of the penis, significant amounts of radioactivity were detected only in the MPG and the S1 DRG, which are the principal ganglia known to innervate the rat penis (Dail et al. 1989).

6.3.4. Role of the GFRα2 coreceptor in the development and/or maintenance of penile nitrergic innervation

The observation that there are defects in some but not all parasympathetic neurons in GFRα2$^{-/-}$ mice led us to study whether the penile nitrergic innervation is affected by ablation of the GFRα2 gene. It was found that GFRα2-deficient mice had a significantly reduced number of nNOS-containing fibers in the dorsal nerves and the crura penis, whereas the penile sympathetic noradrenergic (TH-immunopositive) innervation, which derives from the paravertebral chains (de Groat and Booth 1993), appeared to be normal. Further studies are needed to show whether the decreased nitrergic fiber density in the penis is due to loss of neurons or reduced axonal arborization. Whatever the reason(s), these results provide evidence for a functional role of GFRα2-mediated signaling in penile MPG neurons. This view may for several reasons be correct despite the observation that GFRα2$^{-/-}$ mice are capable of reproduction. First, the present results do not exclude subtle deficits in reproductive performance. Second, it is common that in mice with gene ablation compensatory mechanisms take over the function thought to highly depend on the deleted gene. For example, mice with targeted deletion of the neuronal NOS gene exhibit an increased expression of penile endothelial NOS (Burnett et al. 1996). The compensatory action of other GDNF family members is a possible explanation of the partial presence of penile NOS-containing nerves in GFRα2$^{-/-}$ mice. Our results suggest a compensatory role especially for GDNF, since its preferred coreceptor GFRα1 is expressed in more than half of the penile MPG neurons of adult rat. Although the similarity of penile neurons between rat and mice cannot be assumed, the present results support the idea that GFRα2-mediated signaling is important for both species. Third, trophic support by other neurotrophic factors is also a reasonable possibility (Burgers et al. 1991; Te et al. 1994; Jung et al. 1999).

7. CONCLUSIONS

1) The present results show that Ret and GFRα1 mRNAs are induced in the rat brain in response to systemic administration of kainate. The kainate induced upregulation of GFRα1 mRNA, but not Ret mRNA, was significantly suppressed in hippocampus by the protein synthesis inhibitor cycloheximide. These results suggest that GFRα1 belongs to late responsive genes, as protein synthesis is necessary for its mRNA's induction by kainate. MK-801, a NMDA-receptor antagonist, suppressed the behavioral seizures, but did not significantly suppress elevated levels of Ret or GFRα1 mRNA, suggesting that non-NMDA receptors are responsible for the induction of their mRNAs following kainate-induced seizures.

2) GFRα2$^{-/-}$ mice suffered a nearly complete lack of parasympathetic innervation of the lacrimal and salivary glands, and a 81% reduction in the number of submandibular ganglion neurons. In addition, these mice show clear reduction in the density of the AChE-positive fiber network in the myenteric plexus of their small intestine. The striking similarity of the phenotypes of mice lacking GFRα2 or NRTN, which includes deficits in parasympathetic and enteric neurons, support the idea that these molecules are components of the same ligand-receptor complex, and that GFRα2 serves as the primary coreceptor for NRTN.

3) Both NRTN and GDNF mRNAs are expressed in the shafts of adult rat penises. NRTN mRNA is expressed in penile smooth muscle in close coincidence with the neuron-target contacts, whereas the expression of GDNF mRNA is focused to the subtunical cell layer in the shaft of the penis. In contrast to the expression of GDNF mRNA, NRTN mRNA expression is also seen in several intrapelvic organs. In addition, the penis-innervating as well as most other MPG neurons, express mRNAs for the GDNF family receptor components Ret and GFRα1-3. This supports the idea that in the adult rat certain GDNF family members may be maintenance factors, not only for pelvic parasympathetic, but also for pelvic sympathetic MPG neurons.

4) In the adult rat, penile MPG neurons as well as penile sensory neurons are able to bind, internalize and retrogradely transport to neuronal somata ^{125}I-GDNF and ^{125}I-NRTN injected into the shaft of the penis. The fact that the ^{125}I-labelled GDNF family ligands, with a neurite outgrowth promoting activity comparable to that of the unlabeled parent proteins, were transported in the presence of a 100-fold excess of cytochrome c but not in the presence of a similar excess of parent proteins, suggests that the retrograde transport was mediated by binding to specific receptors on the axon terminals.

5) On histochemical examination of the proximal part of the shaft and the crura penis from wild-type and GFR$\alpha 2^{-/-}$ mice, a significantly different pattern of NADPH diaphorase-positive nerve fibers was noted. GFR$\alpha 2^{-/-}$ mice had a significant reduction in the number of NADPH diaphorase-positive fibers in the dorsal penile nerves. In addition, in the crura penis the number of nNOS-containing fibers was significantly reduced outside the perivascular area. This points to a physiological role for GFRα2-mediated signaling in penile erection-inducing neurons.

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APPENDIX I

Summary of developmental deficits in Ret$^{-/-}$, GF-ligand$^{-/-}$ and GFRα$^{-/-}$ mice. Modified from Baloh et al. 2000.

	RET$^{-/-}$	GFRα1$^{-/-}$	GDNF$^{-/-}$	GFRα2$^{-/-}$	NRTN$^{-/-}$	GFRα3$^{-/-}$
Gross phenotype	Lethal P0	Lethal P0	Lethal P0	Viable	Viable	Viable
CNS						
Dopaminergic						
Substantia nigra	ND	ns	ns	ncd	ncd	ND
Motor						
Trigeminal	ND	22% loss	19% loss	ND	ND	ND
Spinal motor	ND	24% loss	22-31% loss	ND	ND	ND
PNS						
Sensory						
Trigeminal	ND	ns	ns	Responsiveness to NRTN↓ *	GFRα2 neuron ↓#	ns
Nodose	ND	0-15% loss	40% loss	ns	ns	ND
DRG	ND	ns	23% loss	ns	GFRα2 neuron ↓#	ns
Autonomic						
SCG	100% loss	ns	35% loss	ns	ns	100% loss at P60
Ciliary	ND	ND	ND	ncd	50% loss	ND
Submandibular	ND	ND	ND	81% loss	45% loss	ncd
ENS	100% loss in the gut distal to the stomach	100% loss in the gut distal to the stomach		Reduced AChE fiber density and impaired contractile activity of gut		ncd
Other deficits	Agenesis or dysgenesis of the kidney	Agenesis or dysgenesis of the kidney		Growth retardation	(Normal growth)	(Normal growth)
References	Schuchardt et al. 1994	Cacalano et al. 1998; Enomoto et al. 1998	Moore et al. 1996; Pichel et al. 1996; Sanchez et al. 1996	Rossi et al. 1999 (II)	Heuckeroth et al. 1999	Nishino et al. 1999

Reduction of neuron number is expressed as % value. AChE, acetylcholine esterase; ENS, enteric nervous system; ncd, no clear deficit on visual inspection; ND, not determined; ns, difference not statistically significant by neuronal count; P, postnatal day; PNS, peripheral nervous system; SCG, superior cervical ganglion. *Explant and dissociated culture of the trigeminal ganglion of GFRα2-deficient mice exhibited reduced responsiveness to NRTN. #In newborn NRTN-deficient mice the number of GFRα2 mRNA expressing trigeminal neurons is significantly reduced. In adult mice 45% of GFRα2 mRNA expressing neurons are absent from the DRG.