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Neurturin and Glial Cell Line-Derived Neurotrophic Factor Receptor-β (GDNFR-β), Novel Proteins Related to GDNF and GDNFR-α with Specific Cellular Patterns of Expression Suggesting Roles in the Developing and Adult Nervous System and in Peripheral Organs

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Cloning strategies were used to identify a gene termed glial cell line-derived neurotrophic factor receptor-β (GDNFR-β) related to GDNFR-α. *In situ* hybridization was then used to map cellular expression of the GDNF-related trophic factor neurturin (NTN) and GDNFR-β mRNA in developing and adult mice, and comparisons with GDNFR-α and RET were made. Neurturin is expressed in postnatal cerebral cortex, striatum, several brainstem areas, and the pineal gland. GDNFR-β mRNA was more widely expressed in the developing and adult CNS, including cerebral cortex, cerebellum, thalamus, zona incerta, hypothalamus, brainstem, and spinal cord, and in subpopulations of sensory neurons and developing peripheral nerves. NTN colocalized with RET and GDNFR-α in ureteric buds of the developing kidney. The circular muscle layer of the developing intestines, smooth muscle of the urether, and developing bronchiolae also expressed NTN. GDNFR-β was found in myenteric but not submucosal intestinal plexuses. In developing salivary glands NTN had an epithelial expression, whereas GDNFR-β was expressed in surrounding tissue. Neurturin and GDNFR-β were present in developing sensory organs. In the gonads, NTN appeared to be expressed in Sertoli cells and in the epithelium of the oviduct, whereas GDNFR-β was expressed by the germ cell line. Our findings suggest multiple roles for NTN and GDNFR-β in the developing and adult organism. Although NTN and GDNFR-β expression patterns are sometimes complementary, this is not always the case, suggesting multiple modi operandi of GDNF and NTN in relation to RET and the two binding proteins, GDNFR-α and GDNFR-β.

Key words: GDNF; neurturin; GDNFR-α; GDNFR-β; RET; in situ *hybridization; CNS; development; kidney; gastrointestinal tract; gonads; kainic acid; GFRα-1; GFRα-2*

Glial cell line-derived neurotrophic factor (GDNF) (Lin et al., 1993; Unsicker, 1996; Olson, 1997) is a distant member of the TGF-β superfamily, which is expressed in many neuronal and non-neuronal tissues during development and, to a lesser extent, in the adult animal (Schaar et al., 1993; Strömberg et al., 1993; Henderson et al., 1994; Hellmich et al., 1996; Nosrat et al., 1996; Suvanto et al., 1996). GDNF signaling is mediated through a two-component system consisting of a glycosyl-phosphatidylinositol-linked protein termed GDNF receptor-α (GDNFR-α) (Jing et al., 1996; Treanor et al., 1996), which binds GDNF, after which the GDNF–GDNFR-α complex binds to and activates the tyrosine kinase receptor RET (Durbec et al., 1996; Trupp et al., 1996). *In situ* hybridization studies of the three gene products, GDNF, RET, and GDNFR-α, have revealed many examples in which a given set of cells expresses GDNF mRNA, whereas cells on which GDNF is assumed to act express both RET and GDNFR-α (Nosrat et al., 1997; Trupp et al., 1997).

Interestingly, however, there are cases in which the GDNF–RET/GDNFR-α match is not obvious. For instance, GDNFR-α mRNA is present in brain areas such as the developing ventral striatum and the olfactory tubercle as well as in hippocampus, whereas the corresponding expressions of RET mRNA are not found (Nosrat et al., 1997). Moreover, there are instances in which RET, but not GDNFR-α, is found in the brain such as in areas of cerebellum, the olfactory bulb, and the subthalamic nucleus (Trupp et al., 1997). These observations suggest either that the two receptor components may function independently of each other and/or that other ligands and/or receptor components exist. The first alternative may be true for Schwann cells, which express GDNFR-α but not RET (Treanor et al., 1996). In this case, the situation may be analogous to the expression by Schwann cells of the p75 NGF receptor, but not *trk*A, suggesting that Schwann cells produce NGF, which is secreted and binds to the p75 receptors to be presented to growing nerve fibers. Similarly, GDNF, also known to be produced by Schwann cells (Trupp et al., 1997), may be presented to nerve fibers bound to GDNFR-α in the cell membrane.

An alternative explanation to apparent mismatches between GDNF and its receptor components requires the presence of additional gene-related products. So far, other RET-related tyrosine kinase receptors have not been identified, but a protein with a high degree of similarity to GDNF was recently discovered and termed "neurturin" (NTN) (Kotzbauer et al., 1996). The

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Table 1. Distribution of NTN and GDNFR-β mRNA in the CNS and peripheral tissues at selected stages

	GDNFR-β				NTN			
Tissue	E17	P0	P7	AD	E17	P0	P7	AD
Olfactory bulb		++	++	+	−	−	−	−
Cingulate cortex			++	++			++	−
Cortex cerebri			++	++	−	−	+	−
Striatum			−	−	−	−	+	−
Septum			+++	++			+	−
Dentate gyrus	+	+	+	++	−	−	−	−
Habenula		+	+				−	−
Corpus pineale			−				+++	
Amygdala			+					−
Thalamus	+	++	+	+	−	−	−	−
Reticular thalamic nucleus		+++	+++	++			−	−
Zona incerta	+++	+++	+++	+++			−	−
Hypothalamus	+	++	++	+	−	−	−	−
Pituitary gland	−	−				+		++[a]
Colliculi		+	++	++	−	−	+	−
Interpeduncular nucleus			+	++			+	−
Peripeduncular nucleus			+	++				−
Supramamillary nucleus			+	+				−
Tegmental nucleus				+				−
Cochlear nucleus				+				−
Cerebellum	+	+	++	+	−	−	−	−
Brainstem	+	++			−	−		−
Trigeminal ganglia	+++	++	++		−	−	−	
Spinal cord, gray matter	+	++	+	−	−	−	−	−
Dorsal root ganglia	+++	+++	++		−	−	−	
Autonomic ganglia	++	+++			−	−		
Peripheral nerve			+			−		
Inner ear	(+)				++			
Olfactory mucosa	++		++		++	+++	++	
Vibrissae	++		++		++	++	+++	
Salivary glands	+	+	−		++	+	+++	
Harder's glands		(+)	−				+	
Lung	++	+	−	−	+	+		−
Myocardium	−		−	−	+	+	+	−
Heart vessels		+	−	−				−
Intestine	−	(+)	+	−	++	+	+	−
Kidney	−	(+)	−	−	+	+	−	−
Adrenal	−	−	+	−	−	−	−	−
Testis				+++			++	+++
Oviduct				−				+++

Intensity of hybridization signals was semiquantitatively estimated as weak (+), moderate, (++), or strong (+++). Very weak signals were denoted (+). A minus sign indicates that we were unable to detect robust signaling above background levels using our oligonucleotide-based method applied to thin sections. This does not rule out the presence of very low levels of mRNA species.

[a] NTN mRNA as well as GDNF mRNA are found in the intermediate lobe of the pituitary gland.

existence of two (and possibly more) proteins in the GDNF family also suggested that the GDNFR-α (Jing et al., 1996; Treanor et al., 1996)–RET (Durbec et al., 1996; Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996) dual receptor complex would not suffice if receptor-specific actions of GDNF and NTN were to be expected. Therefore, we and others have searched for possible GDNFR-α-related binding proteins. Here we report finding a GDNFR-α-related gene, with ~50% homology to GDNFR-α, which we have termed GDNFR-β. While the present paper was in preparation, three independent studies reported the presence of this same gene, expressed the protein, and called it "TGF-β-related neurotrophic factor receptor 2" (TrnR2) (Baloh et al., 1997), "RETL2" (Sanicola et al., 1997), and "NTNR" (Buj-Bello et al., 1997; Klein et al., 1997), respectively. The different names given to this receptor are reflections of the almost simultaneous and independent discovery of its existence in different laboratories. Most recently it has been suggested that GDNFR-α should be called GFRα-1 and GDNFR-β should be called GFRα-2, but there is still no general agreement (GFRα Nomenclature Committee). The term GDNFR-β finds some support in the fact that both GDNF and NTN appear able to bind to and act through GDNFR-β (Baloh et al., 1997; Sanicola et al., 1997). There is no previous information about the cellular expression of NTN mRNA and only limited information about the

Table 2. Comparison of distribution of GDNF, NTN, RET, GDNFR-α, and GDNFR-β

System	Stage	GDNF	NTN	RET	GDNFR-α	GDNFR-β
DA neurons of substantia nigra and VTA	Developing	−	−	+++	+++	−
	Adult	−		+++	+++	−
Striatum	Developing	++	++	−	+	−
	Adult	(+)	−	−	+	−
Cortex cerebri	Developing	+	+	−	+	++
	Adult	+[a]	−	−	+	++
Cerebellum	Developing	++	−	++	++	++
	Adult	(+)[a]	−	++	++	+
Hippocampus	Developing	+	−		++	++
	Adult	(+)[a]	−	+[a]	++	++
Thalamus	Developing	++	−	++	++	++
	Adult	(+)[a]	−	++	++	++
Hypothalamus	Developing			+	++	++
	Adult	−	−	+[a]		++
Brainstem	Developing	++	++	++	++	++
	Adult	−[a]	−	++	++	++
Spinal cord	Developing	+	−	+++	+++	++
	Adult	−	−	++	++	−
α-Motor neurons	Adult	−	−	+++	+++	−
Sensory ganglia	Developing	−	−	+++	+++	+++
	Adult		−			++
Autonomic ganglia	Developing	−	−	+++	+++	++
	Adult					
Kidney	Developing	+++	++	++	++	(+)
Intestine	Developing	++	++	++	++	+

Symbols are as in Table 1. Data were compiled from the present study and Nosrat et al. (1996, 1997).
[a]Data are from Trupp et al. (1997).

cellular expression of GDNFR-β/TrnR2/RETL2/NTNR-α (Klein et al., 1997; Sanicola et al., 1997). Therefore, to complement existing studies of the distribution of the tyrosine kinase receptor RET and of GDNFR-α performed by us and by others (Nosrat et al., 1997; Trupp et al., 1997), we have also performed a detailed analysis of cellular expression patterns of GDNFR-β in the present study. We additionally compared the distribution of NTN and GDNFR-β mRNA with the expression of GDNF, RET, and GDNFR-α mRNA in adjacent tissue sections to determine the neuroanatomical and histological basis for possible interactions between GDNF and NTN with the three known receptor components.

MATERIALS AND METHODS

Cloning of GDNFR-β sequences

EST homology search. A homology search was performed against the NCBI World Wide Web server dbEST database (http://www.ncbi.nlm.nih.gov/dbEST/index.html) using the sequences for the human and rat GDNFR-α. Homology was found to W73681, W73633, R02135, H12981, H05619, T03342, R02249, and HFC1KA111. Highest homology was found against H12981. The clone was obtained (Genome Systems Inc.) and fully sequenced (1051 bp). Based on this sequence information the following oligonucleotides were produced for *in situ* hybridization: CCCAATCATGCCAGCATAAGAGCCCAGACACGCCTGGTAATT and CTGGATGGCGTTCCGGAGGCATGGGTTCTCGGTGAAGTC CCTGA.

Screening of EST-tagged filters. pAT112 containing a partial fragment of the human GDNFR-α cDNA was digested with *Not*I and *Sph*I that cuts in the MCS flanking fragment. A fragment of ~300 bp was gel-isolated and used as a hybridization probe to screen EST high-density gridded filters (Genome Systems). Twenty-six unique double-positive clones were identified, sequenced, and compared against the human GDNFR-α sequence using a BLAST search. GS21 (corresponds to GenBank accession number H12981) again showed the highest homology of all clones sequenced. The sequences identified by these two approaches were identical to those recently reported by Baloh et al. (1997) and Buj-Bello et al. (1997).

Animals

Embryonic day 17 (E17; $n = 2$), E19 ($n = 2$), and E20 ($n = 2$) and postnatal day 1 (P1; $n = 4$), P7 ($n = 4$), and P14 ($n = 4$) as well as adult ($n = 4$) BALB-c mice (B&K Universal, Sollentuna, Sweden) were used for the *in situ* hybridization mapping studies. Adult mice, litters, and staged pregnant females were kept under standardized light, temperature, and humidity conditions and given food and water *ad libitum*. Adult female 150 gm Sprague Dawley rats were kept under similar conditions and used for the kainic acid experiments. Postnatal and adult mice were killed by cervical dislocation; fetuses and adult rats were killed by decapitation.

In situ *hybridization*

Serial 14 μm sections were used for *in situ* hybridization with oligonucleotide probes for neurturin, GDNF, c-RET, GDNFR-α, and GDNFR-β. Two nonoverlapping oligonucleotide probes complementary to mouse GDNF (50 mer probes from bases 456–505 and 540–589 in the sequence deposited in GenBank, accession number L15305), an antisense oligonucleotide probe complementary to the base pairs 547–596 in the extracellular domain of RET, two different antisense oligonucleotide probes complementary to base pairs 100–149 and 805–851 in GDNFR-α (GenBank, accession number U59486), two different neurturin antisense oligonucleotide probes (50 mer probes from bases 679 and 970, GenBank, accession number U78109), and oligonucleotide probes to the two defined regions of GDNFR-β described above were also synthesized (DNA Technologies, Aarhus, Denmark; and Scandinavian Gene Synthesis, Köping, Sweden). The oligonucleotide probes had no significant similarities to other sequences deposited in GenBank, and each pair generated identical *in situ* hybridization pattern in tissue. A 50 mer random control probe was used as

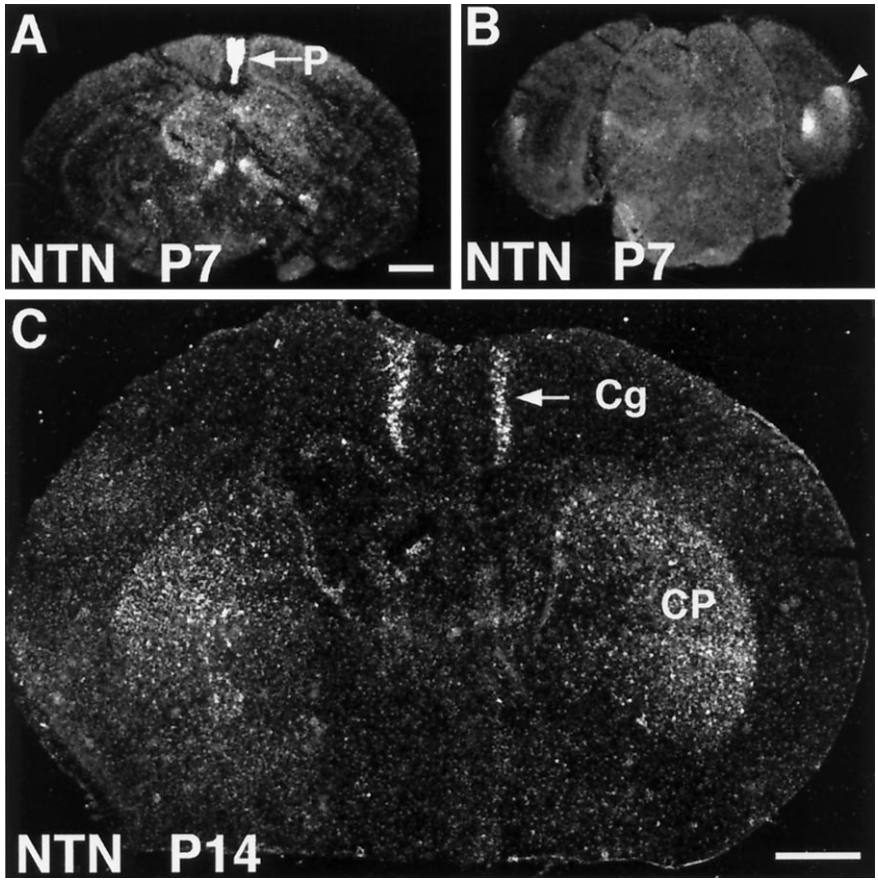

Figure 1. NTN mRNA hybridization signals in the mouse brain during P7 and P14. Expression of NTN mRNA 1 week after birth is seen, e.g., in the central gray (*A*), corpus pineale (*P*) (*A*) and parts of the entorhinal cortex (*B, arrowhead*). Two weeks after birth there is labeling of the caudate putamen (*CP*) and cingulum (*Cg*) (*C*). The caudate putamen appears somewhat diffusely labeled with the NTN probe, and one observes an increasing gradient of labeling laterally. Scale bars: *A, B* (shown in *A*), 1 mm; *C*, 1 mm.

a negative control (Nosrat and Olson, 1995). The *in situ* hybridization protocol using radiolabeled oligonucleotides was according to the method of Dagerlind et al. (1992) (also see Nosrat et al., 1996). After development, the slides were counterstained with cresyl violet or toluidine blue and mounted (Entellan; Merck, Darmstadt, Germany). Photomicrographs of the sections were scanned and digitally processed for brightness and contrast. Microscopically verified artifacts such as dust particles and loosened tissue fragments were retouched.

Specificity considerations. To ascertain specificity of the observed hybridization signals, we used two different nonoverlapping probes for each mRNA species except c-RET. In this case we had previously found that one of our probes, directed toward a sequence coding for the extracellular domain of the protein kinase receptor, displays a considerably higher signal-to-noise ratio than a second probe, directed toward a sequence in the intracellular protein kinase domain. Pairs of probes generated the same hybridization patterns. The GDNF, RET, and GDNFR-α probes have been characterized previously (Nosrat et al., 1996, 1997). All hybridization was performed under high stringency conditions. Tissue sections hybridized with the random probe were processed together with the specific probes.

Positive controls included (1) the use of two nonoverlapping probes for a given mRNA species, (2) observation of correct labeling patterns in known areas when possible, and (3) microscopy performed by two independent experienced observers who agreed on all findings. Negative controls included (1) the inclusion of a random control (2) the fact that specific probes failed to label irrelevant structures, and (3) the different probes functioning as controls for each other, because they had similar GC contents. The specificity of the *in situ* hybridization procedure is dependent on high stringency conditions (in particular the rinsing temperatures) used and the positive and negative controls described above. It can nevertheless not be excluded that the probes also hybridize to unknown but related species of mRNA if such mRNA species exist that have two 50 mer sequences that are both equal, or almost equal, to the chosen areas of our probes.

Detection of positive autoradiographic signals was based on observations of accumulations of silver grains in the emulsion above specific cells and tissues identified by the staining procedures and seen in serially sectioned material. Only cells over which accumulations were clearly above the surrounding background level, detectable using dark-field microscopy and a primary magnification of ≤10×, were regarded as positive. To allow direct comparisons of the distribution of different species of mRNA, serial sectioning and labeling of consecutive sections with different probes was used. All comparisons were thus based on tissues sectioned, hybridized, exposed to emulsion, and further processed together.

RNA isolation and Northern blots

Total RNA was isolated from different frozen tissues of 6-week-old C57/BL mice. Thirty grams of total RNA were electrophoresed in 1% agarose–formaldehyde gels and blotted onto GeneScreen Plus membranes. Membranes were then hybridized with a ^{32}P-labeled GDNFR-β cDNA probe, washed at high stringency (0.1× SSC and 0.1% SDS, 55°C), and exposed to films (Kodak BioMax-MS; Eastman Kodak, Rochester, NY).

RESULTS

Neurturin and GDNFR-β mRNA expression in developing and adult mice

The distribution of NTN and GDNFR-β mRNA in different areas of the CNS and selected other tissues is summarized in Table 1. Table 2 compares selected observations described in Table 1 with what is known about the presence of GDNF, RET, and GDNFR-α mRNA in tissues using *in situ* hybridization. In the following, we shall discuss positive and negative findings from *in situ* hybridization of NTN and GDNFR-β. We will confine our descriptions of NTN and GDNFR-β hybridization patterns in the CNS to gray matter and expression patterns that appear to be neuronal. Methodological constraints associated with nonspecific labeling of white matter tracts for certain oligonucleotide probes limit the ability to study expression in glial elements uniquivocally.

The olfactory bulb

Neurturin mRNA was not found in the olfactory bulb. GDNFR-β mRNA, however, was seen in the olfactory bulb, notably in the mitral cell layer and the internal plexiform layer.

Cortex cerebri, the hippocampal formation, and septum

Neither of our two oligonucleotide probes detected any NTN mRNA in these areas of the prenatal mouse CNS. Two weeks after birth, strong NTN mRNA expression was found in cingulum (Fig. 1*C*), subiculum, and the entorhinal cortex. A thin layer of cells in basal cortex, close to corpus callosum was also NTN mRNA-positive, although at a somewhat weaker level. Labeled cells in subiculum were large nonpyramidal neurons. Robust NTN mRNA signals were not seen in neurons of the adult mouse cortex or hippocampal formation.

GDNFR-β mRNA hybridization was found in cortex cerebri from birth to adulthood, with the strongest signals observed at P14. The cortical GDNFR-β mRNA was found mainly in two layers corresponding to layers 3 and 4 and parts of layers 5 and 6 (Fig. 2). Frontal, parietal, and occipital cortex contained labeled cells laterally down through the temporal association cortex for the outer layer of labeled cells and down to perirhinal cortex for the inner layer of labeled cells. Medial cortex had the most distinct expression.

RET mRNA was seen in P7 medial and parietal cortex, and GDNFR-α mRNA was seen only in medial cortex (Fig. 3*A,C*). The GDNFR-β signals at P7 were mainly observed in cortical layers, which appeared to be intercalated with those of RET mRNA expression (Fig. 3*A,E*).

Neuronal NTN mRNA expression was below our detection limit in the hippocampal formation. GDNFR-β mRNA was found postnatally to be weakly expressed in stratum oriens of CA1 and CA2 of the posterior hippocampus and relatively distinct in the dentate gyrus (Figs. 3*E*, 4*E*, 5*A*, 6*B*). In septum we found NTN mRNA-positive neurons in the lateral parts at P7 and P14. GDNFR-β mRNA signals were found in lateral septum from birth and in all postnatal stages (Figs. 2*A*, 5*A*).

Cerebellum

Neurturin mRNA was not observed in cerebellar neurons. GDNFR-β mRNA hybridization was present in the developing cerebellum from E19 and onward in the Purkinje cell layer (Fig. 6*C*). This signal appeared to peak around P7 and was still present in the adult.

Dopamine systems and the basal ganglia

Neurturin mRNA was expressed in the postnatal striatum (Fig. 1*C,D*). Modest signals were seen at P7, and stronger signals were seen at P14. It appeared as if the expression was neuronal, and at P14 a mediolateral gradient of increasing signal strength was noted. We were unable to see a patch or matrix pattern of NTN mRNA distribution in striatum. GDNFR-β mRNA was not detected in striatum.

Figures 4 and 6, *A* and *B*, compare the expression of RET, GDNFR-α and GDNFR-β receptor components in the substantia nigra area of mesencephalon of adult and newborn mice. Although RET and GDNFR-α mRNA are both clearly present in the A9 and A10 dopamine neurons, as demonstrated previously (Nosrat et al., 1997), consecutive sections hybridized for GDNFR-β revealed that almost no dopamine neurons express this molecule. GDNFR-β mRNA, however, was detected from P0 and onward into adulthood in the substantia nigra area. It was also diffusely distributed in VTA from birth onward. Areas of the interpeduncular nucleus, the adult supramammillary nucleus, and the peripeduncular nucleus were all GDNFR-β-positive (Fig. 4*E,F*). GDNFR-α was also expressed in the adult supramammillary nucleus.

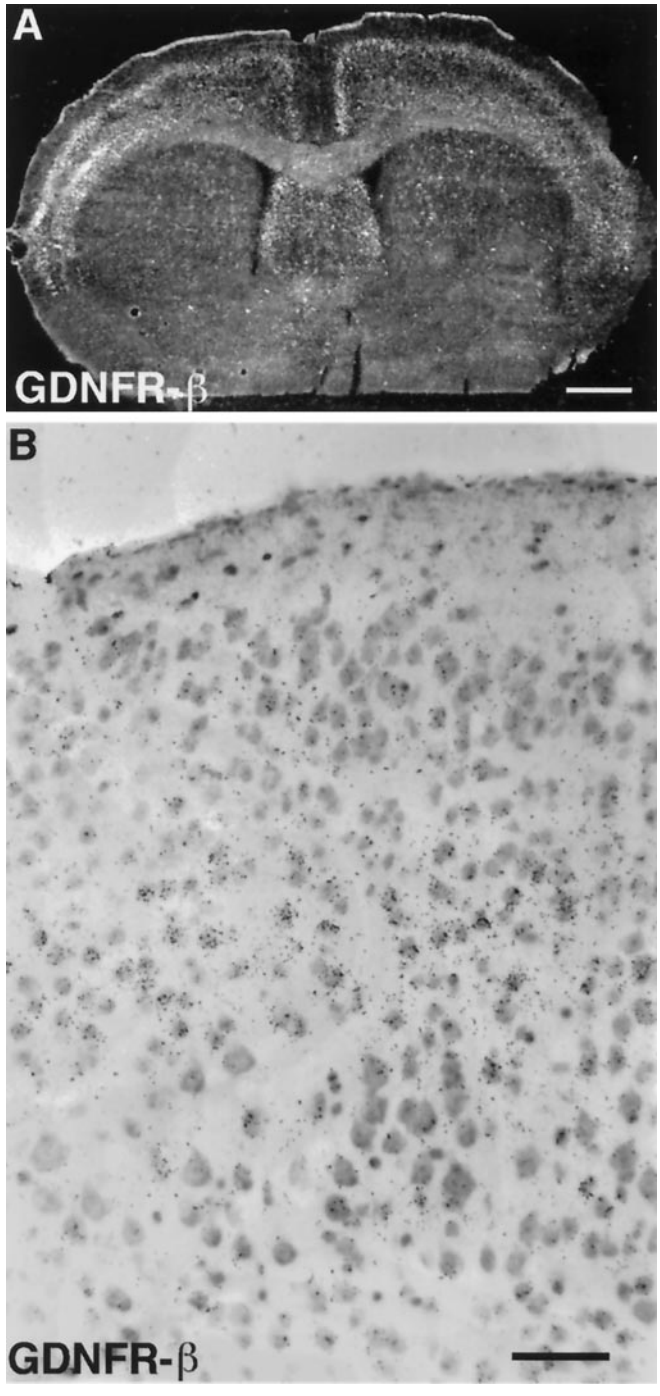

Figure 2. GDNFR-β mRNA signal in adult cortex and lateral septum (*A*, dark-field view). Higher magnification (*B*, bright-field view) reveals signal in parts of the frontal cortex in layers 3 and 4. Scale bars: *A*, 1 mm; *B*, 50 μm.

Thalamus

NTN was not detected. GDNFR-β mRNA was detected in thalamic areas during prenatal development. In the adult thalamus we found labeling in the reticular thalamic nucleus (Fig. 3*E*). This

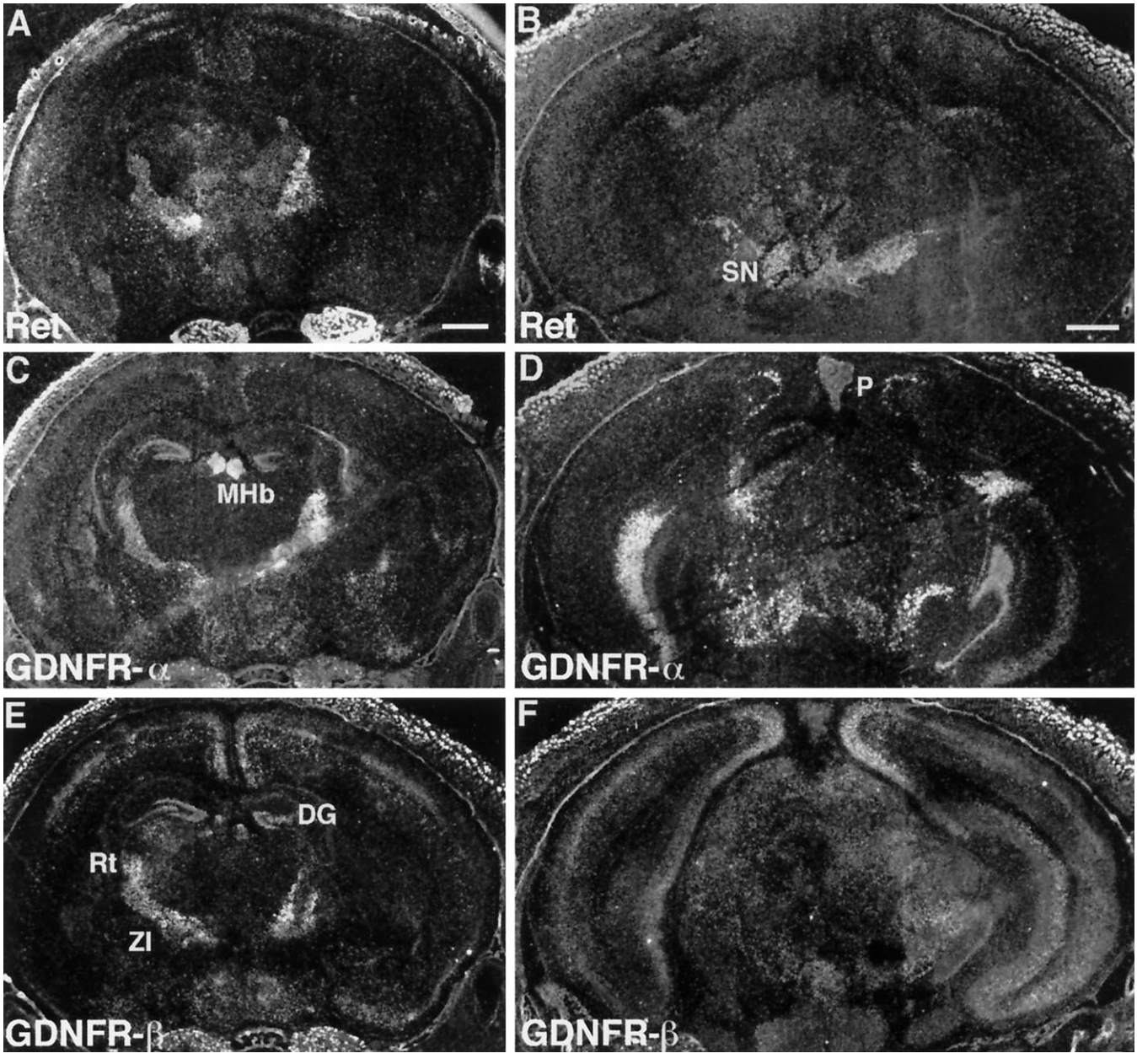

Figure 3. Comparison between prominent RET, GDNFR-α, and GDNFR-β mRNA expression in 1-week-old mouse brain, dark-field view. All three genes show strong expression in zona incerta (*ZI*) and the reticular thalamic nucleus (*Rt*) (*A, C, E*). In the dentate gyrus, a positive signal is seen for GDNFR-α and GDNFR-β (*C, E*), but not for RET. Dopamine neurons of substantia nigra (*SN*) and the ventral tegmental area have a distinct expression pattern of RET and GDNFR-α (*B, D*), whereas GDNFR-β is absent or very weakly expressed (*F*). Cortex cerebri represents another area where the signal distribution differs markedly: GDNFR-β labels two layers in cingulum and frontal and parietal cortices (*E*), GDNFR-α labels only medial cortex (*C*), and RET labels almost the same areas as GDNFR-β, but slightly weaker and in two different cortical layers (*A*). GDNFR-β appears diffusely in many areas in the brainstem, such as the superior colliculus (*F*). Distinct signals for GDNFR-α, but not for RET and GDNFR-β, are seen in the medial habenula (*MHb*), corpus pineale (*P*), and cornu ammonis caudally. The trigeminal ganglion shows signal for GDNFR-α, GDNFR-β, and RET, although strongest for the latter. Scale bars: *A, C, E* (shown in *E*), 100 μm; *B, D, F* (shown in *F*), 100 μm.

nucleus was also GDNFR-α mRNA-positive (Fig. 6*A*); GDNFR-β (Fig. 5*C*) and RET hybridization both revealed distinct signaling in zona incerta at all postnatal stages investigated (Figs 3*A,C,E,* 6*B*). GDNFR-α and GDNFR-β were also positive in the zona incerta area of E17 and E19 fetuses.

Hypothalamus

NTN mRNA was not seen. There were multiple GDNFR-β mRNA-positive areas in hypothalamus at all stages investigated. One area with strong signals was the ventromedial hypothalamic nuclear complex, particularly prominent at P14.

Habenula and pineal gland

A weak GDNFR-β signal was found in the postnatal habenula area. In comparison, GDNFR-α mRNA was strongly expressed in the medial habenular nucleus, whereas RET mRNA was not prominent (Fig. 3*A,C,E*). Interestingly, the developing P7 pineal

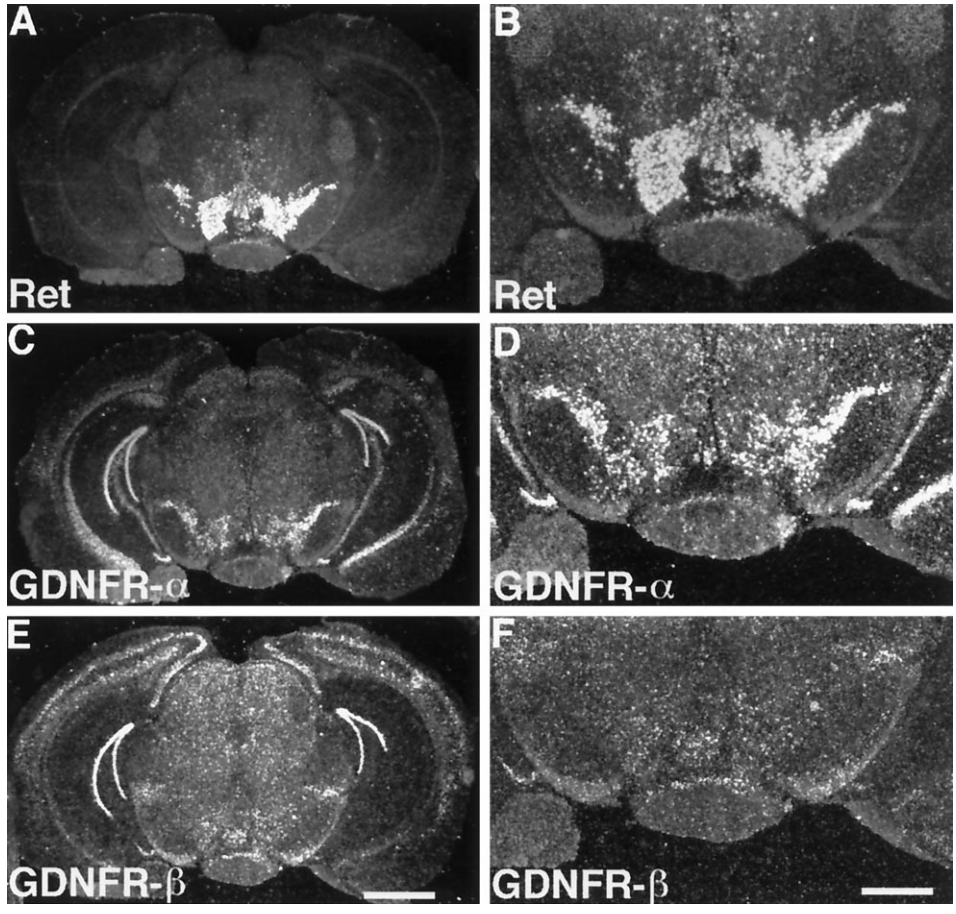

Figure 4. Dark-field photomicrographs depicting distribution of the different GDNF receptor mRNA species in adjacent sections of adult substantia nigra. Both RET and GDNFR-α mRNA give rise to robust hybridization signals in the dopamine neurons of substantia nigra and the ventral tegmental area (*A–D*), whereas GDNFR-β is only weakly expressed in the ventral tegmental area (*F*). All three probes are positive in the supramammillary nucleus (*A–F*). The dentate gyrus is positive for GDNFR-α and GDNFR-β but not for RET mRNA (*A, C, E*). Other areas of GDNFR-β mRNA signal include the peripeduncular nucleus, superior colliculus, and two layers in cortex. Note that in contrast to GDNFR-β, RET and GDNFR-α are not detectable in cortex. Scale bars: *B, D, F* (shown in *F*), 1 mm; *A, C, E* (shown in *E*), 2 mm.

gland was found to express NTN mRNA at a rather high level (Fig. 1*A*).

Brainstem

Neurturin mRNA signals begin to appear in this area around birth. Nuclei in the periaqueductal gray and certain other brainstem areas showed weak signals (Fig. 1*A,B*). Neurturin mRNA hybridization was found in mesencephalon at P7 and P14. Positive cells were also found in the interpeduncular nucleus at P14. GDNFR-α mRNA expression was prominent in the trigeminal motor nucleus and the facial nucleus at P0 (Fig. 6*A*). GDNFR-β mRNA signals were found distributed throughout large areas of the E17–E20 brainstem. Postnatally, GDNFR-β continued to be widely expressed in many brainstem areas (Fig. 6*B*), including the colliculi and the interpeduncular, peripeduncular, cochlear, and tegmental nuclei.

Spinal cord

In contrast to GDNF mRNA, which has been reported to be expressed in the embryonic spinal cord, we did not observe NTN mRNA expression at any stage investigated. GDNFR-β, on the other hand, showed an expression pattern reminiscent of that of GDNFR-α in the spinal cord (Fig. 7*C,D*). At P7 GDNFR-β mRNA hybridization was found in gray matter labeling most neural cells, including α-motor neurons (Fig. 7*C,D*). GDNFR-α at the same stage was strongly positive in motor neurons and more weakly expressed in other neuronal elements (Fig. 7*C*). GDNFR-α, in contrast to GDNFR-β, was also expressed in the adult spinal cord.

Peripheral nervous system

Neurturin mRNA was not found in any ganglia investigated. GDNFR-β mRNA, on the other hand, was strongly expressed at all stages studied in a subpopulation of neurons of both dorsal root ganglia (small- to medium-sized neurons; Fig. 7*A*) and the trigeminal ganglion (medium to large neurons; Fig. 6*B*). The pattern of GDNFR-β expression in dorsal root ganglia appeared not to overlap that of GDNFR-α (Fig. 6*A*), whereas RET mRNA was expressed in the vast majority of ganglion perikarya. Postnatal superior cervical ganglia also had strong GDNFR-β-positive neurons at all investigated stages. In peripheral nerves we did not see NTN mRNA expression during development. Neonatal peripheral nerves were strongly positive for GDNFR-β mRNA (Fig. 8*E*).

Kidney and ureter

Figure 9 shows consecutive sections of the E19 kidney hybridized for all five known genes in the GDNF families of factors and receptors. Interestingly, NTN mRNA is found in the developing buds of the metanephric ducts (Fig. 9*B*). GDNFR-β was not found in the mesenchyme or in the developing epithelial components. Weak GDNFR-β signals were associated with central pelvic regions of the developing kidney (Fig. 9*E*). The smooth muscle layer of the E20 urether expressed a strong NTN mRNA signal.

Gastrointestinal tract

During development, NTN mRNA was found exclusively in the circular layer of the external smooth muscle layer both prenatally

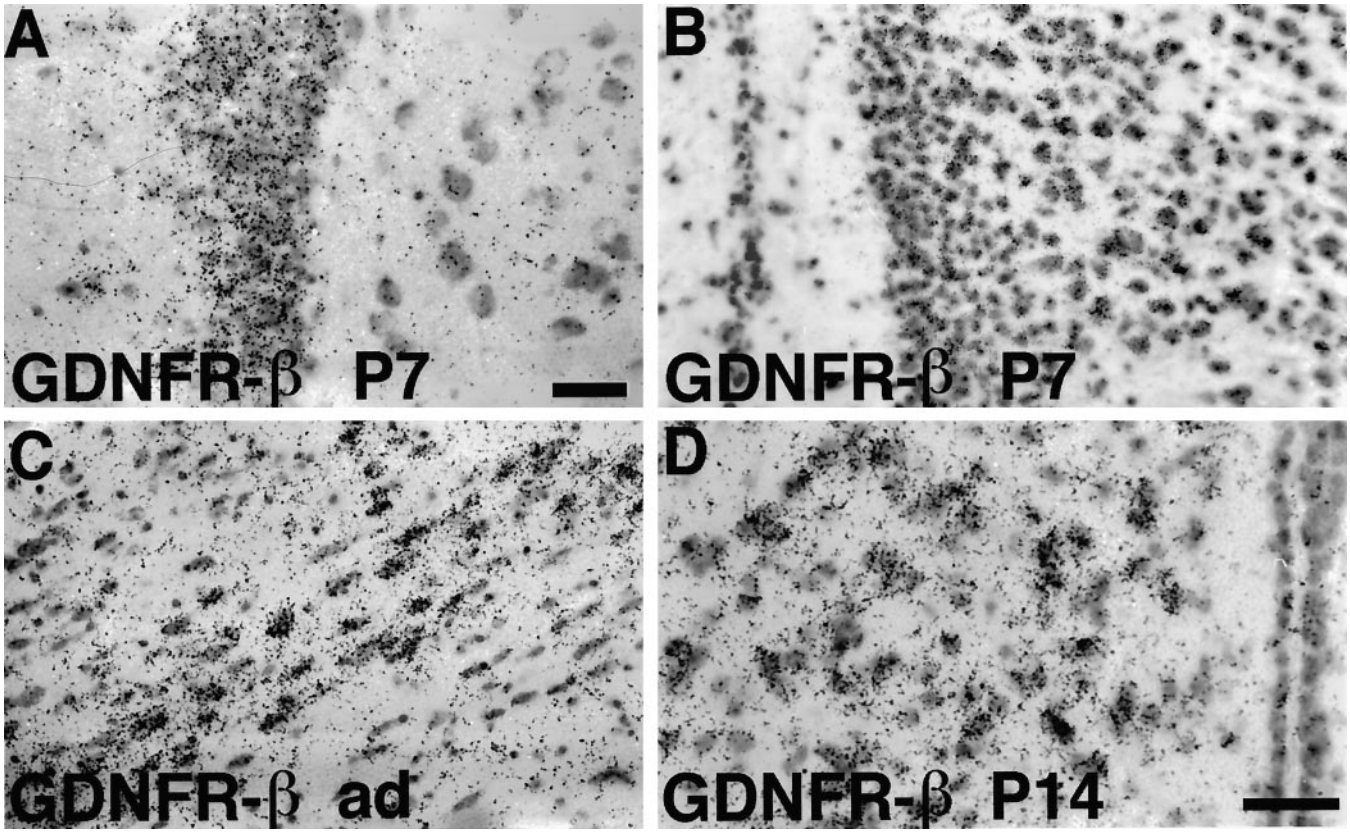

Figure 5. High-magnification bright-field photomicrographs of some of the most prominent neuronal GDNFR-β hybridization signals in the brain. *A,* The lateral dentate gyrus in a 1-week-old mouse shows distinct hybridization signal. *B,* In cingulum at the same stage several layers are positively labeled. *C,* A distinct signal for GDNFR-β mRNA is found in the adult zona incerta. *D,* The lateral septum in the 2-week-old mouse also shows strong labeling. Scale bars: *A, B, C* (shown in *A*), 50 μm; *D,* 50 μm.

and postnatally but not in the adult. During the same developmental period, GDNFR-β was expressed in the myenteric plexus of Auerbach but not in the submucous plexus of Meissner. These patterns of expression therefore differ from those of GDNF mRNA, which is present in both the circular and longitudinal outer smooth muscle layers, and of RET and GDNFR-α mRNA, which are both present in the myenteric as well as the submucous plexuses.

Exocrine glands

Interestingly, NTN and GDNFR-β mRNA were both present in the developing salivary glands (Fig. 10*A,B*). Although NTN appeared to be present in the epithelial portions of the glands, GDNFR-β had a more peripheral and therefore complementary expression in the interstices of the glandular tissues. These expression patterns were seen in the submandibular gland and the parotid gland. Additionally, such complementary labeling patterns were seen in the lingual glands of von Ebner and other small salivary glands of the oral cavity. Neurturin mRNA expression was also found in the Harderian gland of the orbit at P0 and P7. GDNFR-β was not seen at P7 in the Harderian gland.

Sense organs

In the developing retina, weak expression of NTN mRNA was found in the pigment cell layer at P7. GDNFR-β mRNA was found in the retina at P0 and P7 located in different cell types. In the inner ear there were clear-cut NTN mRNA signals from E17 to E20 and a weak signal at P0. GDNFR-β was weakly expressed at E17 and E19.

In the olfactory mucosa, NTN mRNA was found in the developing glands of Bowman, whereas robust GDNFR-β mRNA expression was found immediately below the olfactory epithelium (Fig. 8*A,C*). In vibrissae, GDNFR-β as well as NTN mRNA was expressed during development (Fig. 8*B,E*). GDNFR-β mRNA was also present around lower parts of hair follicles during development.

Striatal musculature

A weak NTN mRNA signal was found in the E17 myocardium. Neurturin continued to be expressed at a low level throughout prenatal development in heart musculature. The expression was somewhat stronger during the first two postnatal weeks and included the auricle. GDNFR-β mRNA was not seen in the developing heart except for a low signal intensity in vessel walls. Neurturin mRNA was not observed in skeletal muscle.

Lung

A weak to moderate NTN mRNA signal was noted in the E17–E20 lung. GDNFR-β mRNA was present at higher levels from E17 to E20 and at low levels at P0. Although NTN mRNA was located in the walls of the bronchiolar system, GDNFR-β mRNA was expressed in the surrounding developing lung parenchyma.

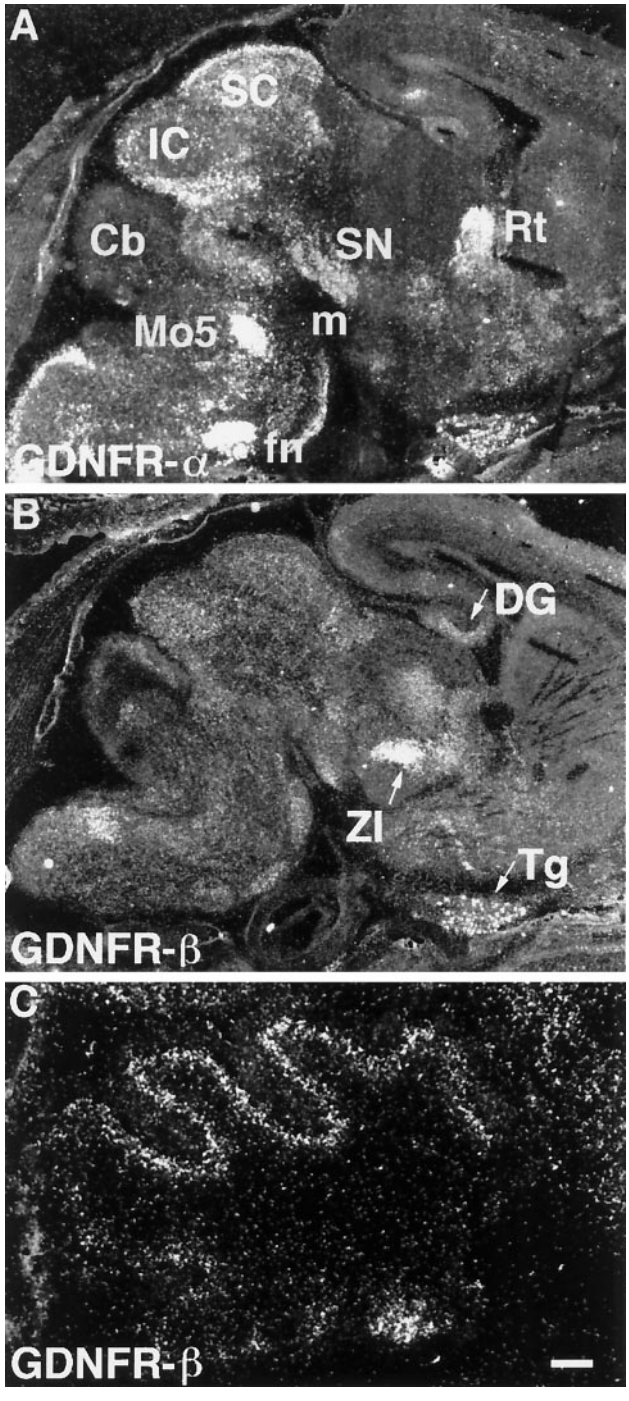

Figure 6. Dark-field images depicting GDNFR-α and GDNFR-β mRNA expression in neonatal mouse CNS. Prominent hybridization of the GDNFR-β probe is seen in zona incerta, dentate gyrus, the trigeminal ganglia, and the superior and inferior colliculus (*B*). No robust GDNFR-β signal is observable in the substantia nigra area (*SN*) in contrast to GDNFR-α (*A*). Cerebellum is positive for GDNFR-β, especially in the Purkinje cell layer (*C*). *Cb*, Cerebellum; *DG*, dentate gyrus; *IC*, inferior colliculus; *fn*, facial nucleus; *m*, mesencephalic flexure; *Mo5*, trigeminal motor nucleus; *SC*, superior colliculus; *TG*, trigeminal ganglion; *ZI*, zona incerta. Scale bar (shown in *C*): *A, B,* 250 μm; *C,* 125 μm.

Adrenal gland

Neurturin mRNA was not noted at E20. GDNFR-β mRNA was not found in the developing adrenal at E17 or E20. At P7, however, there was a GDNFR-β signal in the developing zona glomerulosa.

Gallbladder

Neurturin and GDNFR-β mRNA appeared to have a complementary distribution in the perinatal gallbladder. Thus, NTN mRNA was found in the epithelium at moderate levels at E20, whereas GDNFR-β mRNA was found under the epithelium at P7 and P14.

Pituitary gland

NTN mRNA was seen in the P0 pituitary gland (Fig. 1*A*). GDNFR-β mRNA was not noted at E17, E19, or P0.

Gonads

A relatively strong GDNFR-β signal was found in the E19 gonads. Interestingly, the adult testis displayed a strong NTN mRNA signal (Fig. 10*C,D*), which appeared confined to the Sertoli cells, whereas the germ cell line displayed a strong GDNFR-β signal in a mosaic of tubuli seminiferi contorti (Fig. 10*E,F*). Neurturin mRNA was also strongly expressed in the oviduct.

Teeth

Both NTN and GDNFR-β mRNA signals were detected in the developing teeth.

Thymus

A weak GDNFR-β signal was noted in the P0 thymus.

Northern blots

Northern analysis was used to confirm hybridization of our probe to specific mRNA species. As can be seen in Figure 11, single bands of the appropriate size were seen in lung and brain tissue from 6-week-old mice, in accordance with the *in situ* hybridization findings in these tissues. Similarly, the lack of signal in kidney is in accordance with the lack of *in situ* hybridization in this tissue. The lack of a positive band in intestine in all probability reflects the low sensitivity of this method compared with *in situ* hybridization, which found a signal restricted to only one ganglionic plexus.

DISCUSSION

To understand neurotrophic signaling and to delineate the roles of neurotrophic factors and their receptors in the organism, it is imperative to determine precisely not only which tissues but which specific cells express the factors and receptors at hand. The discovery of neurturin, a protein with neurotrophic properties (Kotzbauer et al., 1996) and 42% amino acid homology to GDNF (Lin et al., 1993; Unsicker, 1996) prompted this first *in situ* hybridization study of NTN mRNA expression patterns to complement the patterns of expression of GDNF as described by us and others (Strömberg et al., 1993; Hellmich et al., 1996; Nosrat et al., 1996; Suvanto et al., 1996).

Although further members of the GDNF family of trophic factors, as well as further members of the dual component receptor families, may well be discovered, the five gene products presently known allow for a series of different possible combinations of receptor components and trophic factor exposure. Theoretically, the three known receptor components could be present in cells in seven different combinations. To provide a histological substrate for these possibilities, we have used consecutive sections to localize by radioactive *in situ* hybridization mRNA generated by all five known genes in the GDNF family of factors and the corresponding receptors. Interestingly, several of the theoretical combinations of receptors noted above appear to be substantiated by the experimental findings. Thus dopamine neurons and α-

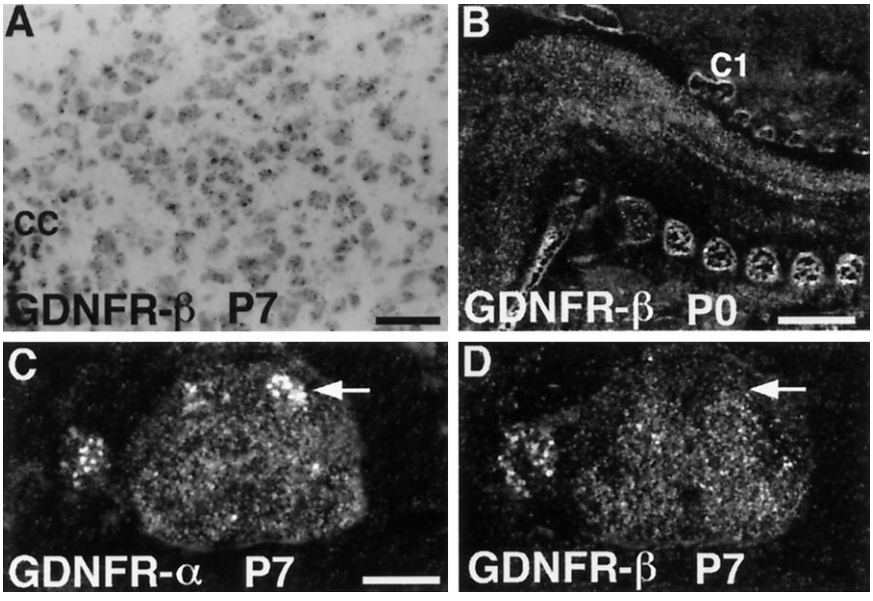

Figure 7. GDNFR-β mRNA hybridization in developing spinal cord. Hybridization is observed in most neurons of the spinal cord during development (bright-field, *A*; and dark-field, *B, D*) and in a subset of neurons in the dorsal root ganglia (dark-field image, *D*). The positive labeling is continuous from medulla oblongata and downward at birth as shown in sagittal section (*B*). One week later (P7) GDNFR-β mRNA signals are still present throughout gray matter, labeling most neurons, including α-motor neurons (*A*, *D*). Compared with GDNFR-α (*C*), which has a similar expression pattern, the most striking difference is the relatively weak expression of GDNFR-β mRNA in α-motor neurons (compare *arrows* in *C*, *D*). *CC*, Canalis centralis. *C1*, First cervical vertebrae. Scale bars: *A*, 50 μm; *B,* 125 μm; *C, D* (shown in *C*), 250 μm.

motor neurons have RET and GDNFR-α, similarly, ganglion cells have RET and GDNFR-α or GDNFR-β, possibly all three receptors. In striatum there appear to be cells that express GDNFR-α but not RET. Similarly, Schwann cells appear to express GDNFR-α and GDNFR-β but not RET. Our findings, therefore, permit a series of speculations as to the functions of the novel proteins NTN and GDNFR-β in the CNS and in the peripheral nervous system, as well as in peripheral organs.

Many unique patterns of expression of NTN mRNA were found both within and outside the CNS, suggesting that NTN has many roles in the developing and adult organism different from those of GDNF. In the cerebral cortex, for instance, NTN mRNA is expressed postnatally in cingulum, subiculum, and areas of entorhinal cortex. Interestingly, GDNFR-β mRNA was expressed in cortical layers that appeared intercalated with those of RET mRNA expression. Although NTN levels were not detected in the adult cortices, GDNFR-β mRNA continued to be expressed in the adult mouse, suggesting that the GDNFR-β ligand in adult mouse cortex might not be NTN. Moreover, GDNFR-β, but not NTN, mRNA was found in the developing and adult cerebellum, again suggesting that GDNFR-β might not function together with NTN in this system.

Neurturin, like GDNF (Strömberg et al., 1993), is expressed in the postnatal developing striatum. We did not find GDNFR-β mRNA in this area; neither was there any expression of RET mRNA, whereas ventral striatum does express moderate levels of GDNFR-α (Nosrat et al., 1997). Taking known receptors into account, it therefore appears that NTN could not exert trophic actions within striatum but, rather, could be a target-derived trophic factor for a striatal input system. The mesencephalic dopamine neurons projecting to striatum express RET and GDNFR-α but not GDNFR-β mRNA. Hence, NTN could influence these neurons via their RET–GDNFR-α combination. It is, however, also possible that NTN could have trophic roles for the corticostriatal input.

In general, GDNFR-β is much more widely expressed in brain areas, both temporally and spatially, than NTN mRNA. For instance, signals in several areas in thalamus and hypothalamus, including very prominent GDNFR-β mRNA expression in zona incerta, were detected. Together these observations suggest that GDNFR-β subserves roles other than mediating NTN effects in many areas of the CNS. This observation also holds true for the spinal cord, in which GDNFR-β mRNA expression was seen in most neural cells of gray matter during development, whereas we did not detect NTN mRNA. Although GDNFR-β levels decreased below the detection level in adult mice, GDNFR-α mRNA expression remained.

In sensory ganglia, distinct populations of neurons expressed GDNFR-β. Our results suggest that populations of dorsal root ganglia cells expressing GDNFR-α and -β are mainly nonoverlapping, and that both the GDNFR-α- and GDNFR-β-expressing cells may express RET. However, a more detailed analysis of ganglia is needed to determine these relationships and the possible existence of cells coexpressing both binding proteins. It is interesting to note that peripheral nerves are strongly positive for GDNFR-β mRNA during development, suggesting that Schwann cells, known to produce GDNF (Hammarberg et al., 1996), express both binding proteins but not RET.

Several observations regarding the role of NTN and GDNFR-β in peripheral organs were also made. The kidneys were of great interest, because both GDNF and RET knock-outs manifest renal agenesis or severe malformations (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Interestingly, NTN mRNA was colocalized with RET and GDNFR-α mRNA in the developing buds of the metanephric kidney, and these buds are embedded in the peripheral mesenchyme, which is strongly GDNF mRNA-positive. GDNFR-β was not present in these outer parts of the developing kidney, suggesting that there is both a mesenchymal–epithelial interaction between GDNF in the mesenchyme and the receptor components RET and GDNFR-α in the epithelial buds, as well as a local autocrine or paracrine effect of NTN, acting via the same set of receptors within the epithelial buds.

We also found interesting patterns of expression of NTN in smooth muscles. Thus, the prenatal developing urether expressed strong NTN mRNA signals in its smooth muscle layers. Similarly, NTN mRNA was expressed in the walls of the bronchiolar tree of the developing lungs, presumably in the smooth muscle cells. In the gastrointestinal tract we have previously demonstrated that GDNF mRNA is expressed by both the circular and the longitu-

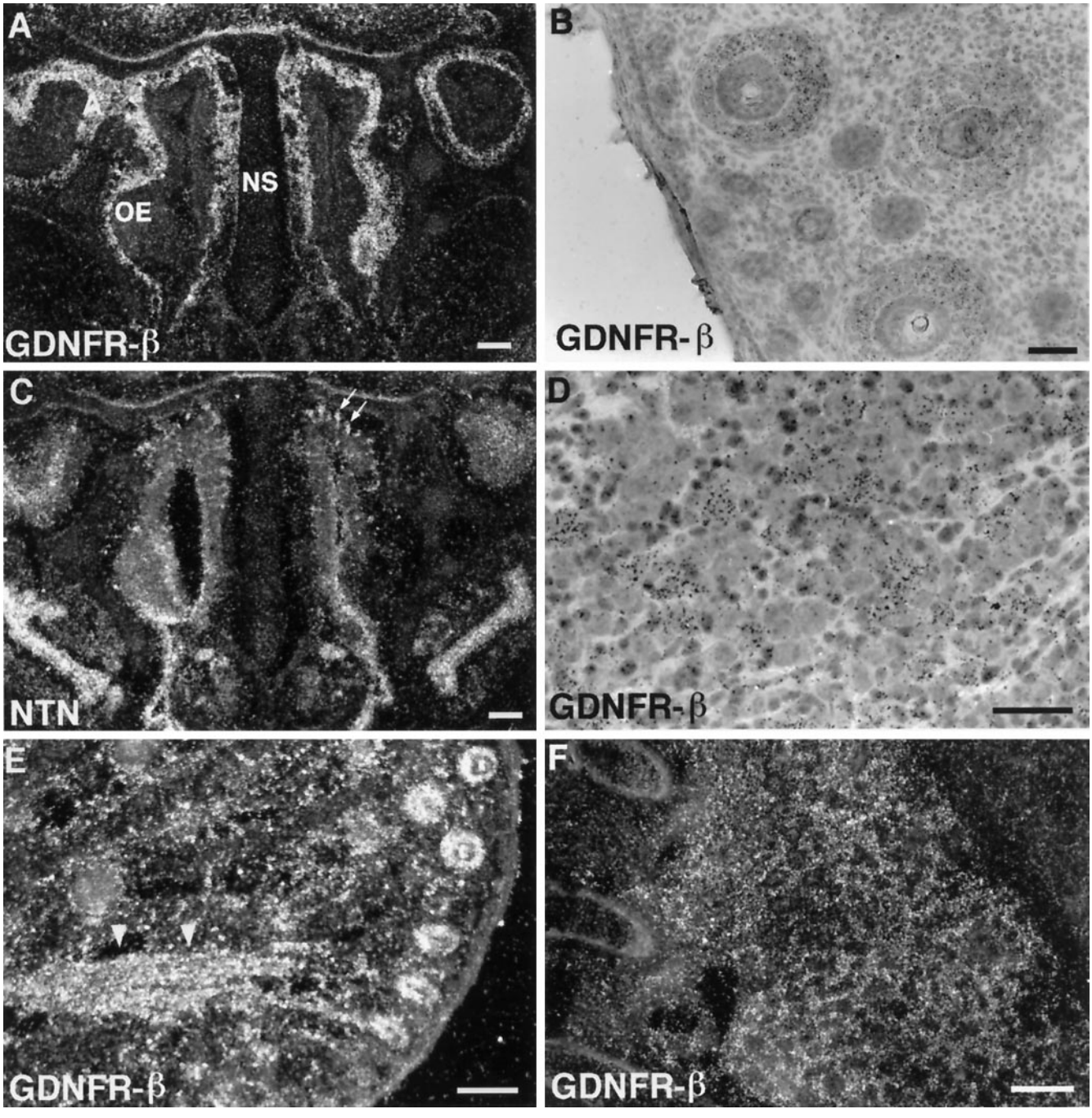

Figure 8. Examples of NTN and GDNFR-β mRNA hybridization in the P0 trigeminal system (*A*--*E*) and of GDNFR-β mRNA in the E20 lung. In the olfactory epithelium NTN and GDNFR-β mRNA appear in complementary patterns (dark-field, *A, C*). NTN mRNA is found in the olfactory epithelium, especially in Bowman's glands (*C, arrows*), and GDNFR-β is found in lamina propria of the olfactory mucosa, a tissue containing abundant nerves and vessels. Vibrissae are positive for GDNFR-β (bright-field image, *B*; dark-field image, *E*), as are peripheral nerves exemplified by nervus maxillaris (*E, arrowheads*). *D*, A subpopulation of neurons in the trigeminal ganglion are positively labeled for GDNFR-β mRNA. In the developing lung GDNFR-β is found in the stromal tissue (dark-field, *F*). Scale bars: *A*, 500 μm; *B*, 50 μm; *C*, 500 μm; *D*, 50 μm; *E*, 250 μm; *F*, 200 μm.

dinal layers of the outer muscle layer, whereas RET and GDNFR-α are both expressed by the myenteric plexus of Auerbach and the submucous plexus of Meissner. We now show that NTN mRNA is expressed exclusively in the circular layer of the external smooth muscle layer both prenatally and postnatally. Moreover, GDNFR-β mRNA is expressed in the myenteric but not the submucosal plexus of the intestinal wall. These observations may help explain how different sets of nerve fibers may innervate the circular versus the longitudinal muscle layers of the gastrointestinal tract and how the two neuronal plexuses of the intestines may be differentially influenced by trophic factors.

The striated musculature of the developing myocardium expressed a low level of NTN mRNA prenatally, and somewhat stronger expression was seen during the first 2 postnatal weeks in the myocardium, including musculature of the auricles. The presence of NTN mRNA in heart striated musculature may be related

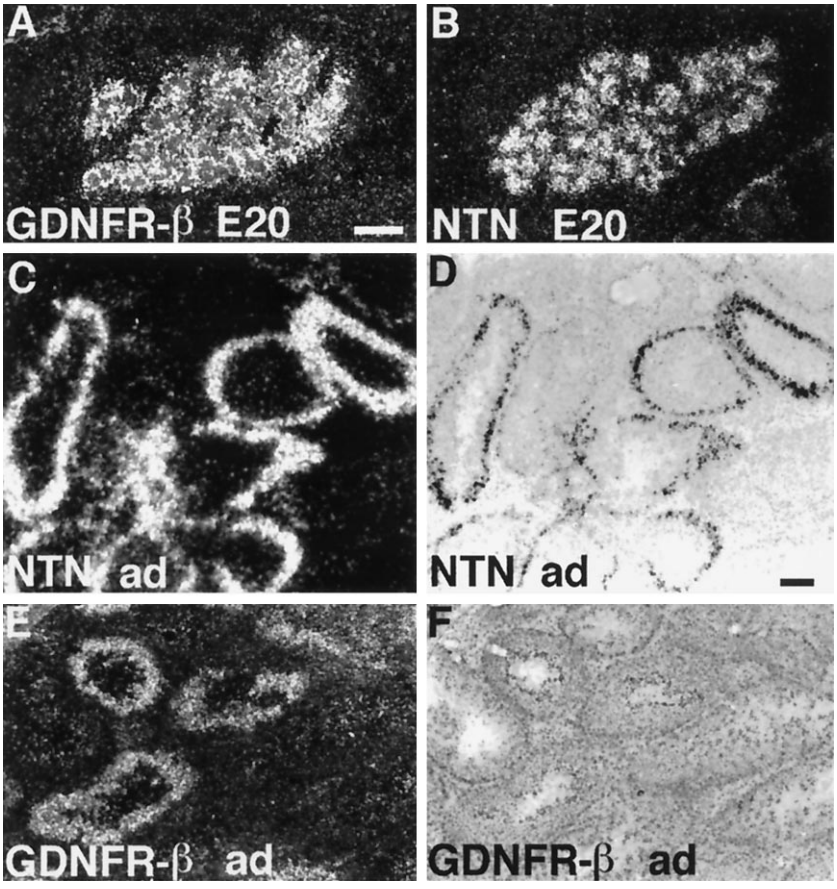

Figure 9. E20 salivary glands and adult testis. Neurturin and GDNFR-β mRNA show a complementary expression pattern in both organs. The developing tubules in the salivary glands are labeled by NTN hybridization (*B*), whereas GDNFR-β mRNA is represented in the stroma surrounding the epithelial structures (*A*). In testis a very strong neurturin mRNA signal is seen in the periphery of some of the seminiferous tubules, suggesting localization to the Sertoli cells (*C*, dark-field view; *D*, bright-field view). GDNFR-β is also expressed in some of the tubules, but at levels indicating localization to the germ cells (*E, F*). Scale bars: *A, B,* 200 μm; *C–F*, 100 μm.

to the developing innervation of the heart and sets this type of striated musculature apart from skeletal striated musculature, in which NTN mRNA was not found at any stage.

Members of the GDNF families of factors and receptors are important in sensory systems. In this study we found NTN mRNA to be expressed in the pigment cell layer of the 1-week-old retina, and GDNFR-β mRNA was found in other cell types of the retina. Both NTN and GDNFR-β expression patterns were also found in the developing inner ear and in the olfactory mucosa. NTN mRNA was expressed in the developing glands of Bowmann, whereas GDNFR-β was expressed in the submucosa. In the vibrissae, a prominent and important sensory organ in rodents, both NTN and GDNFR-β mRNA was strongly expressed during development.

Salivary glands represent another interesting example of a complementary pattern of expression of NTN and GDNFR-β mRNA. During development NTN appears to be produced by the epithelial portions of the glands, whereas GDNFR-β appears to be present in the surroundings. The degree of resolution of the radioactive *in situ* hybridization method does not permit us to determine whether the GDNFR-β expression is localized to the myoepithelial cells and/or interstitial tissue outside of the basal membranes of the glands. Not only major salivary glands but also small salivary glands of the oral cavity, such as the glands of von Ebner, displayed this complementary pattern of mRNA expression.

In developing teeth NTN and GDNFR-β mRNA were both expressed. The complex development of the teeth and the changing pattern of expression of these two genes will be dealt with elsewhere.

Finally, we have found a very interesting pattern of expression of NTN and GDNFR-β in the gonads and the female genital system. Thus it appears as if NTN mRNA is expressed by the Sertoli cells, whereas the germ cell line expresses the GDNFR-β receptor. Because NTN mRNA was also found to be expressed by the epithelium of the oviduct, it is conceivable that sperm may be influenced first by NTN from the Sertoli cells and, after reaching the female genital tract, by NTN produced by the oviduct.

Although only described briefly in the present study, we found that the immune system and the endocrine system also show patterns of expression of the GDNF families of factors and receptors. Thus both NTN and GDNFR-β have been noted in the developing pituitary gland. Strikingly, the 1-week-old pineal gland was found to express NTN mRNA at a rather high level. We have previously demonstrated that the pineal gland also expresses GDNF mRNA (Ebendal et al., 1995).

In conclusion, the complex and widespread patterns of expression of NTN and GDNFR-β mRNA and the relation of these expression patterns to those of GDNF, RET, and GDNFR-α suggest that the roles of the GDNF family of proteins and its receptor components are many fold in both the developing and adult organism, and both within and outside the nervous system. These relationships may be analogous to those noted for the nerve growth factor families of trophic proteins and receptors, in which it is now realized that the neurotrophins exert a plethora of functions in the organism, including roles in the immune system, the endocrine system, and the reproductive system. Germ line as well as somatic mutations of members of the GDNF families of factors and receptors are known (Mulligan et al., 1993; Edery et al., 1994; Romeo et al., 1994; Angrist et al., 1996; Ivanchuk et al., 1996). Both loss of function and gain of function mutations of RET have been described. It is not unlikely that additional human

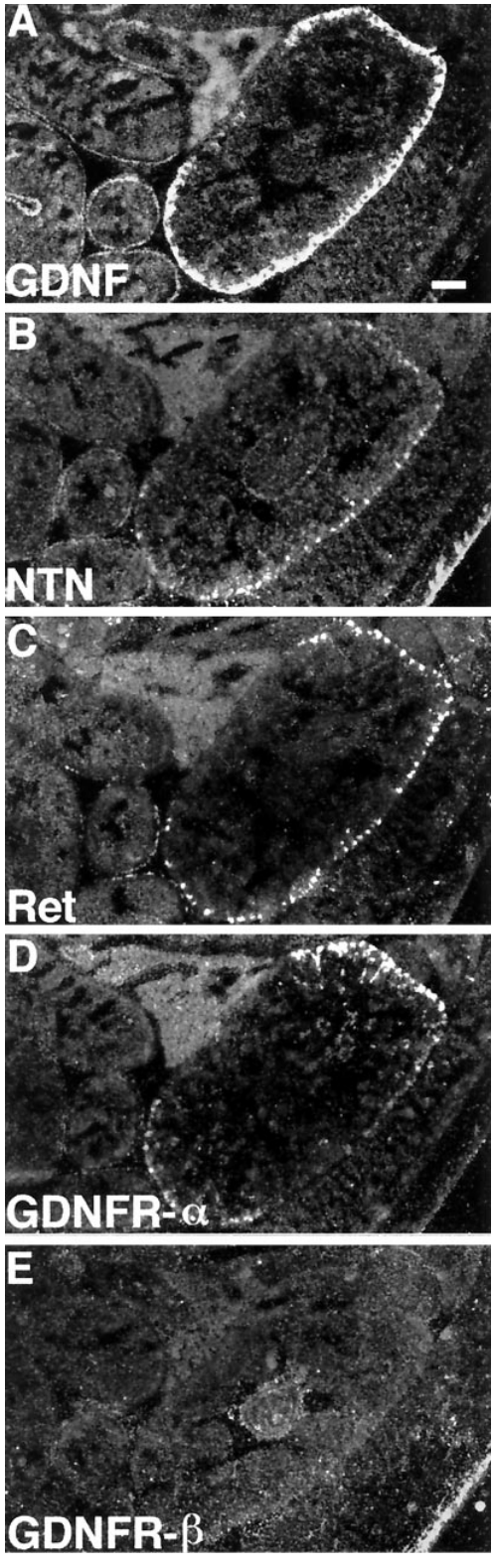

Figure 10. Dark-field photomicrographs showing the mRNA distribution of all known GDNF family members in the developing kidney at embryonal day 19 (*A–E*). NTN mRNA is expressed in the developing epithelial buds. GDNF mRNA, in contrast, is localized in peripheral mesenchyme. GDNFR-α and RET show similar patterns of probe labeling in the peripheral epithelial buds, whereas GDNFR-β could not be detected in the periphery. Weak GDNFR-β as well as weak NTN hybridization signals were found in pelvic regions of the developing kidney. Scale bar (shown in *A*), 500 μm.

Figure 11. Northern blot analysis of GDNFR-β levels in postnatal week 6 mouse tissues. Total RNA from indicated tissues (30 gm/slot) is shown. A 1 kb ^{32}P-labeled GDNFR-β cDNA probe was used for hybridization. Note single bands.

mutations of one or several of the five known gene products may exist in which the manifestations may be explained by their expression in the organism as described here.

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