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CNS glia are targets for GDNF and neurturin

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Abstract Glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) are two closely related growth factors reported to selectively act on distinct neuronal populations in the CNS. Both GDNF and NTN signal through a receptor complex consisting of the signal transducing subunit, Ret, and a ligand-specific binding subunit, termed GDNF family receptor (GFR)α-1 and GFRα-2, respectively. By using RT-PCR, we observed that mRNAs encoding the subunits of both receptor complexes are widely expressed throughout the developing brain, suggesting the presence of targets for these growth factors other than the ones known today. We provide evidence that these targets include glial cells.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) was originally isolated from a CNS glial cell line in a screen for survival promoting factors for midbrain dopaminergic neurons (Lin et al. 1993). GDNF is 42% homologous in its amino acid sequence to neurturin (NTN), a recently isolated growth factor (Kotzbauer et al. 1996). Both GDNF and NTN signal through a receptor complex consisting of the tyrosine kinase receptor, Ret, and a novel class of glycosyl-phosphatidylinositol-linked proteins, namely GDNF family receptor (GFR)α-1 (also referred to as GDNFR-α or TrnR1), and GFRα-2 (originally termed NTNR-α or TrnR2), respectively. (Jing et al. 1996: Treanor et al. 1996; Baloh et al. 1997; Buj-Bello et al. 1997; Klein et al. 1997). The α-subunits show a selective high-affinity binding for GDNF and NTN, respectively, thus allowing for a ligand-specific activation of Ret (Buj-Bello et al. 1997; Klein et al. 1997). The only NTN-responsive cells in the CNS identified so far are midbrain dopaminergic neurons and spinal cord motor neurons (Klein et al. 1997; Horger et al. 1998). In addition to dopaminergic midbrain neurons, established GDNF targets in the CNS are cerebellar Purkinje cells (Mount et al. 1995) and noradrenergic neurons of the locus coeruleus (Arenas et al. 1995), as well as facial nerve and spinal cord motor neurons (Oppenheim et al. 1995; Yan et al. 1995). However, additional cells responsive to GDNF and NTN seem to exist within the CNS. For example, in contrast to the limited number of known GDNF targets, mRNA encoding GDNF is widely expressed within the perinatal and adult rodent brain (Springer et al. 1994; Choi-Lundberg and Bohn 1995; Pochon et al. 1997). To identify these targets, we have studied the expression of mRNAs encoding the subunits of the GDNF and NTN receptor complexes in various brain regions by RT-PCR. We further determined whether GDNF and/or NTN affect only neurons or non-neuronal cells as well.

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Materials and methods

Animals and collection of brain tissue

Sprague-Dawley rats were purchased from Charles River Wiga, and mated for 15 h. Pregnant dams were killed by CO_2 inhalation on gestational day 15 or 17 (day of insemination=embryonic day 0). Embryos were removed by Cesarean section and their brains were collected. In addition, brains were obtained from rat pups at postnatal days (P) 1, 8, and 15 (day of birth=P0). In all cases, cortex, striatum, diencephalon, mesencephalon, and pons were removed under sterile conditions as described previously (Engele and Bohn 1992). Tissue pieces were either collected in ice-cold PBS for cultivation or were immediately frozen in liquid nitrogen for RT-PCR analysis.

Cell cultures

To establish glial cultures, P1 cortex, diencephalon, striatum, mesencephalon, or pons were incubated in Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS (Gibco) containing 0.1% trypsin and 0.02% EDTA. After 20 min, trypsin action was terminated by transferring tissue pieces to Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (Gibco) supplemented with 10% fetal calf serum (FCS; Gibco). The

Table 1 RT-PCR primers and predicted product length. (*GDNF* Glial cell line-derived neurotrophic factor, *GFR* GDNF family receptor, *NTN* neurturin, *SNAP25* synaptic vesicle associated protein)

mRNA	Primer sequence	Primer length	Product length
GDNF	5′-GAA GTT ATG GGA TGT CGT GGC-3′	21 bp	401 bp
	5′-CGT AGC CCA AAC CCA AGT CAG-3′	21 bp	
GFRα-1	5′-CCA GAG TCA AGG TCT GTC AGC-3′	21 bp	359 bp
	5′-GGG GAT CTC ATT CTC AGA CCC-3′	21 bp	
Ret	5′-GCA CAG CTC TGC TCT ATG TCC-3′	21 bp	188 bp
	5′-GAG CTG CTC CCA GGA ACT ATG-3′	21 bp	
NTN	5′-CAG CTC CCT GCT ATC TGT CTG-3′	21 bp	191 bp
	5′-CAG CCC AGG GAG AAA GTT CTC-3′	21 bp	
GFRα-2	5′-GTC CCC AGC GAG TAT ACC TAC-3′	21 bp	575 bp
	5′-CAG GTG GTG ATG ACA CTG GTC-3′	21 bp	
CD31	5′-TGG TGG GCT TGT CTG TGA ATG-3′	21 bp	376 bp
	5′-CTG TCC TGC TCT GTC TCG GGT-3′	21 bp	
SNAP25	5′-TGG ATG AGC AAG GCG AAC AAC-3′	21 bp	384 bp
	5′-TGG ATG AGC AAG GCG AAC AAC-3′	21 bp	
β-actin	5′-CTA CAA TGA GCT GCG TGT GGC-3′	21 bp	271 bp
	5′-CAG GTC CAG ACG CAG GAT GGC-3′	21 bp	

tissue was dissociated by trituration and undissociated pieces were removed by passing the suspension through a 50-μm nitex mesh. Cells were then pelleted by centrifugation at 400 g for 5 min and resuspended in culture medium, consisting of 90% minimal essential medium (Gibco) and 10% horse serum (HS; Gibco). Viable cells that excluded trypan blue were counted with a hematocytometer and seeded at a density of 40 000 cells/cm^2 into 48-well cluster plates (Costar) coated with poly-D-ornithine (Sigma). Cultures were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Upon reaching confluency, cells were trypsinized and replated at lower densities. Replating was repeated at least 3 times before cultures were used for experiment. The culture medium was renewed every 2nd day.

The glial cell line, Mes42 (Engele et al. 1996), and the neuronal cell line, B104 (Schubert et al. 1974), were propagated in Dulbecco's minimum essential medium (DMEM; Gibco) supplemented with 5% HS and 5% FCS. The neuroblastoma line, Neuro2, was expanded in DMEM containing 10% FCS.

RNA isolation and RT-PCR assay

Total RNA was isolated by acidic guanidium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi 1987) and contaminating DNA was digested with DNase I (1 U/μl; Boehringer). Total RNA concentration was measured by spectrophotometric absorbance at 260 nm. Fifty micrograms of RNA were used as the substrate for reverse transcription (M-MLV; Gibco; 400 U/50 μl). The cDNA obtained was amplified in a final volume of 50 μl with 3 μl cDNA, 1.5 U *Taq* DNA polymerase, 1×PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, and 20 pmol of both sense and antisense primers. Amplification was carried out with 20–35 PCR cycles of 93°C for 1 min, 63°C for 1 min, 62°C for 1 min, 61°C for 1 min, and 72°C for 2 min. In all cases, the PCR was also performed on non-reverse transcribed RNA to control for contaminating genomic DNA. Each cDNA preparation was normalized to the level of β-actin message. The primers used and the expected size of the reaction products are given in Table 1. GDNF primers were designed to recognize both the full length GDNF mRNA as well as an alternatively spliced transcript carrying a 78-bp deletion in the preproprotein sequence, as described by Choi-Lundberg and Bohn (1995). The identity of the amplified products was either confirmed by restriction enzyme analysis as specified in the text or by nucleotide sequencing using the dideoxy technique and an ABI 377 sequencer.

Immunocytochemistry

Cultures were fixed with phosphate-buffered (4%) paraformaldehyde for 30 min and permeabilized with saponin (0.1%; 10 min). Cells were then incubated for 24 h at 4°C with antibodies against the astrocytic marker, glial fibrillary acidic protein (GFAP; 1:1250; Accurate). Antibody staining was visualized using the Vectastain ABC kit (Vector Laboratories) and 3,3′diaminobenzidine as chromagen.

Results

The mesencephalic glial cell line, Mes42, as well as the neuronal cell lines, B104 and Neuro2A, were used to establish and validate RT-PCR assays for mRNAs encoding GDNF, GFRα-1, Ret, NTN, and GFRα-2. RT-PCR of Mes42 cells with GDNF primers usually yielded one band representing the full length GDNF mRNA (Fig. 1). Only occasionally a second and much fainter band, corresponding to the alternatively spliced product, was present. In addition to GDNF, Mes42 cells also expressed mRNA encoding GFRα-1. RT-PCR of mRNA isolated from B104 cells with either NTN or GFRα-2 primers resulted in the amplification of products of the appropriate size (Fig. 1). A specific product corresponding to Ret could be amplified from RNA obtained from Neuro2A cells. To confirm the identity of the amplified products, bands were eluted with the QuiaEx II kit (Quiagen) according to the manufacturer's instructions and incubated for 1 h at 37°C with one of the following restriction enzymes: *Hin*fI (GDNF, GFRα-1), *Sau*3A (Ret), or *Ava*II (NTN). The resulting fragments were seperated on a 2% agarose gel and visualized by ethidium bromide staining. In all cases, digestion of the RT-PCR products resulted in fragments of the predicted size (data not shown). In the case of GFRα-2, the identity of the PCR product was confirmed by nucleotide sequencing (data not shown).

The expression of GDNF and the GDNF receptor complex was studied in the neocortex, striatum, diencephalon, mesencephalon and pons of embryonic day (E) 15, E17, P1, and P8 rats. This time window was chosen since it represents an extremely active phase of neuronal development. In all experiments, cDNA was normalized to β-actin mRNA levels (Fig. 2A). For initial qualitative analysis, PCR assay was performed at 35 cycles. In all

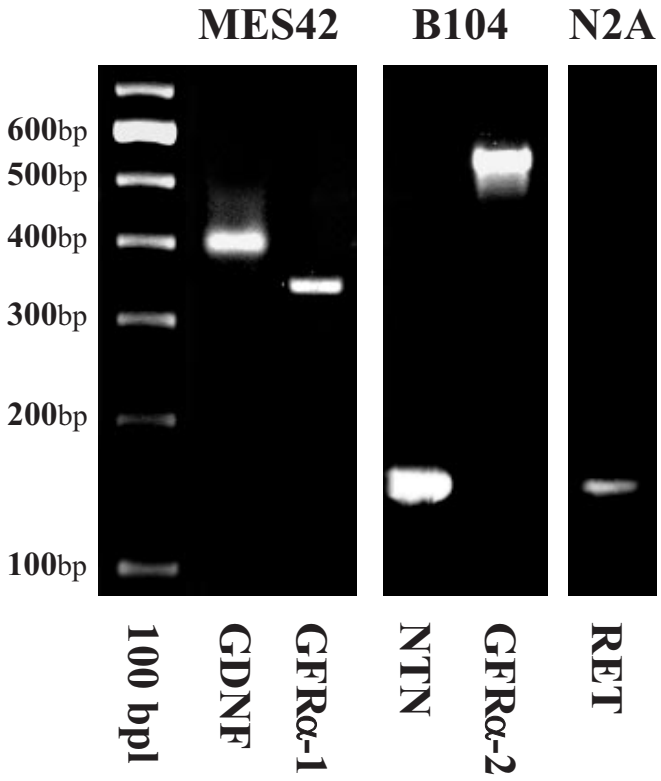

Fig. 1 RT-PCR of total RNA isolated from the cell lines Mes42, B104, and Neuro2A (N2A) with primers for glial cell line-derived neuotrophic factor (GDNF), GDNF family receptor (GFR)α-1, Ret, neurturin (NTN) and GFRα-2. (*bpl* Base pair ladder)

brain areas examined, mRNA encoding GDNF was undetectable up to E17 and was expressed thereafter throughout the brain (Fig. 2B). A similar widespread expression applied to mRNAs encoding GFRα-1 and Ret. Moroever, in all brain areas the expression of both GFRα-1 and Ret mRNAs clearly preceded that of GDNF and were already detectable at E15. Since Ret is also activated by NTN via binding to GFRα-2, we examined the expression of mRNAs encoding NTN as well as GFRα-2. Both NTN and GFRα-2 transcripts were present throughout the brain as early as E15 (Fig. 2). To further determine whether quantitative differences exist in the expression levels of the various receptor subunits between different brain areas and time points, analysis was performed at different PCR cycles. mRNA levels were considered high, moderate, or low when the specific PCR product was first detectable after 20, 25, or 35 PCR cycles, respectively (Table 2). Moderate levels of mRNA encoding Ret and GFRα-1 were present in the embryonic striatum, diencephalon, mesencephalon, and cortex with no apparent changes up to the 1st postnatal week. In contrast, both Ret and GFRα-1 mRNA levels were low in the embryonic cortex and increased to moderate levels postnatally. At E15, all brain areas contained low levels of GFRα-2 mRNA. At P1 moderate levels of GFRα-2 transcripts were present in the cortex, striatum, diencephalon, and mesencephalon while levels remained low in the pons. These findings reveal a widespread expression of both the GDNF and NTN receptor complexes within the developing brain, thus, essentially confirming our prediction of the presence of additional targets for GDNF and NTN within the CNS.

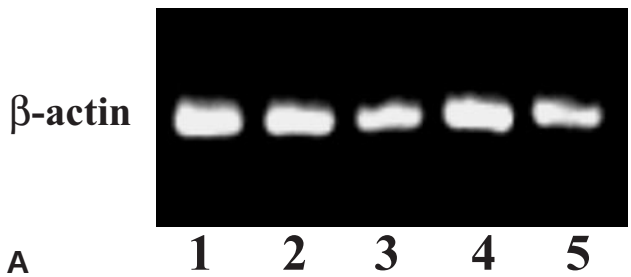

Fig. 2 **A** RT-PCR analysis of various postnatal (P) day 1 brain areas for β-actin, serving as an internal standard. *Lane 1* Cortex, *lane 2* diencephalon, *lane 3* mesencephalon, *lane 4* pons, *lane 5* striatum. **B** RT-PCR of total RNA isolated from various brain regions with GDNF, GFRα-1, Ret, NTN, and GFRα-2 primers. *Lanes 1, 5, 10, 15* Cortex, *lanes 2, 6, 11, 16* diencephalon, *lanes 3, 7, 12, 17* mesencephalon, *lanes 4, 8, 13, 18* pons, *lanes 9, 14, 19* striatum, *lane 20* water blank. Size of the reaction products is given in base pairs. Note that in all brain regions, RT-PCR with GDNF primers predominantly results in the amplification of a product of approximately 400 bp corresponding to the full length GDNF mRNA. Also note that levels of PCR products encoded by the alternatively spliced GDNF transcripts (323 bp) are extremely low or absent

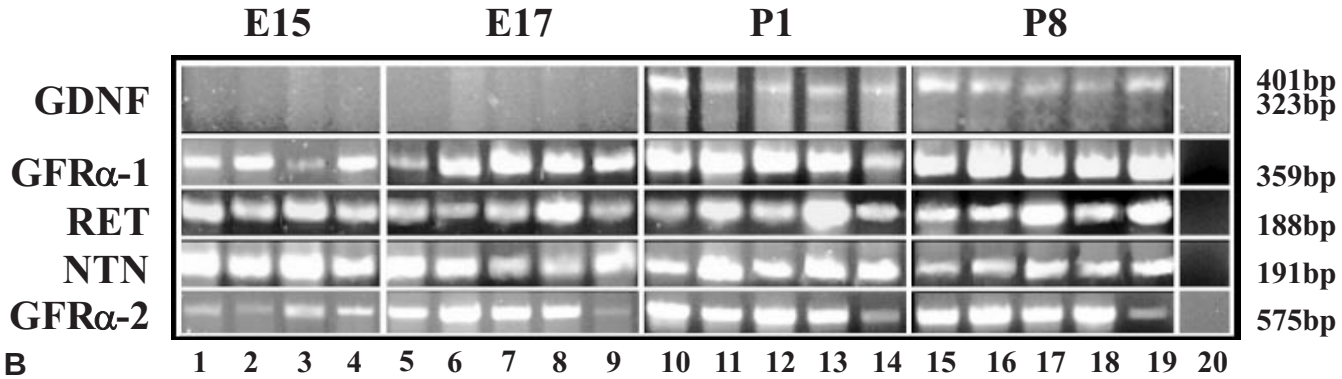

Table 2 Semiquantitative analysis of GDNF and NTN receptor complex expression in the developing brain. Transcript levels were considered, moderate (++) or low (+) when the specific product was first detactable after 25, or 35 PCR cycles, respectively. (*o* not tested, *E* embryonic day, *P* postnatal day)

Brain region	Age	Transcript		
		Ret	GFRα-1	GFRα-2
Cortex	E15	+	+	+
	E17	+	+	o
	P1	+	+	++
	P8	++	++	o
	P15	o	o	++
Striatum	E15	o	o	+
	E17	++	++	o
	P1	+	+	++
	P8	++	++	o
	P15	o	o	+
Diencephalon	E15	++	++	+
	E17	++	++	o
	P1	++	++	++
	P8	++	++	o
	P15	o	o	++
Mesencephalon	E15	++	++	+
	E17	++	++	o
	P1	++	++	++
	P8	++	++	o
	P15	o	o	++
Pons	E15	++	++	+
	E17	++	++	o
	P1	++	+	+
	P8	++	++	
	P15	o	o	++

Initial attempts to identify cells expressing the functional GDNF receptor complex within the various brain regions by immunocytochemically phenotyping neurons that respond to GDNF by c-fos expression confirmed the known neuronal targets for GDNF, but did not reveal any additional targets (Engele and Schilling 1996; B. Franke and J. Engele unpublished observations). To address the possibility that the GDNF receptor complex is expressed by glia, glial cultures were established from various regions of the P1 brain and maintained with serum-supplemented culture medium. Under these conditions few neurons survive. Remaining neurons were eliminated by replating cultures 2 or 3 times prior to analysis. The glial nature of the cultured cells was confirmed by immunocytochemistry for the astrocytic marker, GFAP. In P1 cultures derived from various brain regions, over 90% of the cells were immunoreactive for GFAP (Fig. 3). The vast majority of the GFAP-immunoreactive (GFAP-IR) cells had a large flat soma with multiple thick protrusions. These cells represent type-1 astrocytes. Some GFAP-IR cells (1%) exhibited a stellate morphology and resembled type-2 astrocytes (Raff et al. 1983). To determine whether the GFAP-negative cells represent other glial cell types, e.g., glial precursors or non-glial cells, cultures were analyzed for mRNAs encoding the endothelial marker, CD31 (DeYoung et al. 1995), and the neuronal marker, synaptic vesicle associated protein 25 (SNAP25; McMahon and Sudhof 1995). In both cases, RT-PCR yielded only a very faint band of the appropriate reaction product (Fig. 4). This confirms that the established cultures predominantly consist of different glial cell types and contain only very few non-glial cells. By RT-PCR, specific products corresponding to GDNF and NTN could be amplified from all regional glial cultures (Fig. 5). Moreover, irrespective of their origin, all glial cultures also contained mRNA encoding Ret and GFRα-1 (Fig. 5). GFRα-2 transcripts

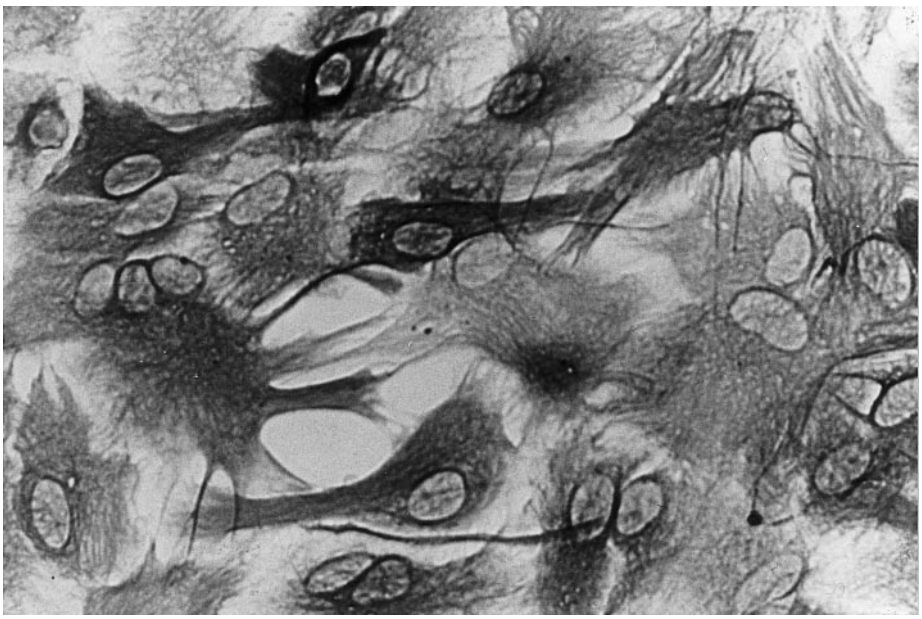

Fig. 3 GFAP-immunoreactive astrocytes in replated cultures of the P1 striatum. Magnification 350×

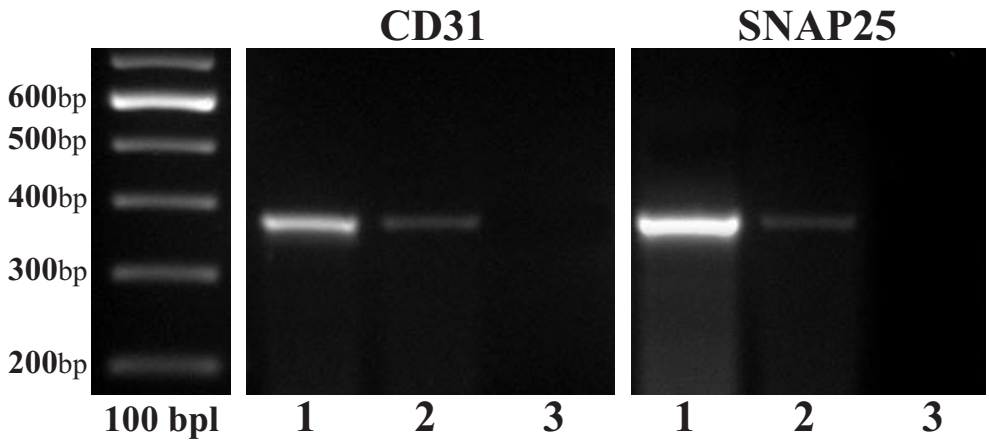

Fig. 4 RT-PCR analysis of glial cultures for the expression of the endothelial marker, CD31, and the neuronal marker, SNAP25. *Lane 1* Positive control (muscle in case of CD31 and P1 cortex in case of SNAP25), *lane 2* glial cultures of the P1 cortex replated 3 times, *lane 3* water blank. (*bpl* Base pair ladder)

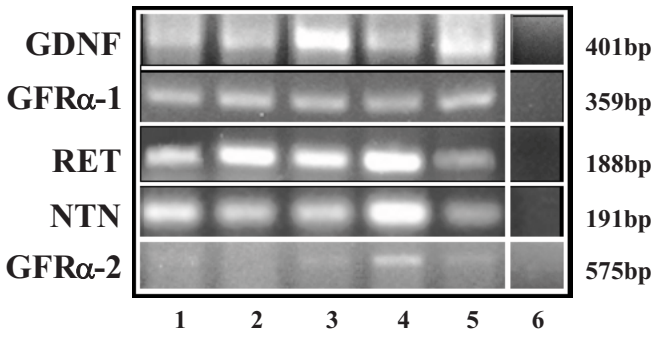

Fig. 5 RT-PCR of glia cultured from various postnatal brain regions with GDNF, GFRα-1, Ret, NTN, and GFRα-2 primers. *Lane 1* cortex, *lane 2* diencephalon, *lane 3* mesencephalon, *lane 4* pons, *lane 5* striatum, *lane 6* water blank. Size of the reaction products are given in base pairs

were present at low levels in glia cultured from the cortex, mesencephalon, pons, and striatum, but were below detection limit in pontine glia.

Discussion

GDNF and its homolog, NTN, are presently regarded as survival factors for selected subpopulations of neurons including midbrain dopaminergic neurons, motor neurons and sensory neurons (Lin et al. 1993; Kotzbauer et al. 1996; Horger et al. 1998). In the present study, we have analyzed the expression of mRNAs encoding the GDNF and NTN receptor complexes in various brain areas by RT-PCR. We have observed that GDNF and NTN receptor complexes, consisting of the shared transducing subunit, Ret, and the ligand-specific binding subunits, GFRα-1 and GFRα-2, respectively (Jing et al. 1996; Treanor et al. 1996; Buj-Bello et al. 1997; Klein et al. 1997), are expressed in the developing cortex, striatum, diencephalon, mesencephalon, and pons. We further demonstrate that in all these brain areas mRNAs encoding the various receptor subunits are present within glia. This identifies CNS glia as a target for GDNF and NTN.

The observation that GFRα-1, GFRα-2, and Ret are expressed in the diencephalon and various parts of the brain stem is in agreement with previous in situ hybridization studies (Nosrat et al. 1997; Widenfalk et al. 1997; Glazner et al. 1998; Yu et al. 1998). In extension of these studies, our present RT-PCR analysis reveals that transcripts encoding both Ret and GFRα-2 are also expressed in the cerebral cortex and striatum, brain areas which do not exhibit hybridization signals for these receptor subunits (Widenfalk et al. 1997; Glazner et al. 1998). The expression of Ret in the cerebral cortex is further underlined by the recent observation that GDNF rescues corticospinal neurons from axotomy-induced cell death (Giehl et al. 1997).

In all the brain areas examined, transcripts encoding NTN and its receptor complex were present as early as E15. This confirms previous predictions of a crucial role for this growth factor during brain development (Widenfalk et al. 1997). Interestingly, throughout the brain, the expression of mRNA encoding GDNF lagged at least 2 days behind that of the GDNF receptor complex which was first detectable at E17. Since NTN also shows low-affinity binding properties for GFRα-1 (Jing et al. 1997), this could indicate that NTN serves as a ligand for both the GDNF and NTN receptor complex during early embryonic development. Alternatively, the observation could reflect the existence of other GDNF-related proteins with high affinity binding properties for the GDNF receptor complex within the CNS. An additional member of the GDNF family of growth factors is persephin. The studies currently available suggest that persephin does not act via Ret (Milbrandt et al. 1998) and, thus, does not represent a ligand for the GDNF receptor complex during early embryonic development.

Independent of the examined brain area, transcripts encoding the GDNF and NTN receptor complexes were detectable in glia. One exception is pontine glia which does not contain detectable levels of GFRα-2 mRNA. Since evidence for the glial expression of the various receptor complexes came from RT-PCR analysis of RNA isolated from cultured glia, it was crucial to determine whether the outcome of these experiments could have

been obscured by non-glial cells "contaminating" the cultures. We have addressed this issue by analyzing the established glial cultures for the presence of the endothelial marker, CD31 (DeYoung et al. 1995), and the neuronal marker, SNAP25 (McMahon and Sudhof 1995). Both markers are expressed at high levels in the respective cell types. RT-PCR assay for these markers resulted in the amplification of very faint bands, suggesting that the cultures contained only very few contaminating neurons and endothelial cells. Taken into account that the cellular expression levels of both CD31 and SNAP25 are much higher than the expression levels of the respective growth factor receptors, it is feasible to assume that these few non-glial cells did not interfere with PCR analysis for the GDNF and NTN receptor complexes. From the observed expression of the GDNF and NTN receptor complexes in cultured CNS glia no conclusion is initially possible as to whether this expression would also occur under normal in vivo conditions. Evidence for such an in vivo expression comes from studies with Schwann cells which express GFRα-1 in vitro (Treanor et al. 1996; Baloh et al. 1997). Similarly, GFRα-1 and/or GFRα-2 are/is detectable in the sciatic nerve as well as in the roots of spinal nerves.

In addition to the various GDNF and NTN receptor subunits, all glial cultures contained transcripts encoding GDNF and NTN. These findings suggest that glia do not only represent a target, but also a source of GDNF and NTN. Since the examined glial cultures consisted of both type-1 and type-2 astrocytes as well as glial precursors, it remains to be established whether glial-derived GDNF and NTN have an autocrine or a paracrine function on CNS glia. We are currently addressing this issue by determining whether the NTN/GDNF receptor complexes and their corresponding ligands are expressed by the same or different glial cell types. In addition to the autocrine/paracrine effects on glia, glial-derived GDNF and NTN might also affect neurons known to respond to either one of these growth factors such as midbrain dopaminergic neurons or pontine locus coeruleus neurons (Lin et al. 1993; Arenas et al. 1995).

The expression of the GDNF and NTN receptor complexes by CNS glia does not necessarily exclude the existence of additional, as yet unknown, targets for GDNF and NTN within the examined brain areas. In other experiments, we have been unable to identify additional target neurons for GDNF using GDNF-induced c-fos expression as a readout for functional signal transduction coupling (B. Franke and J. Engele unpublished observations). This approach does not, however, consider the possibility that GDNF might affect neurons in a c-fos-independent manner. The observation that both NTN and its receptor complex are expressed very early during embryonic development may further indicate that such additional targets are neural precursor cells. In conlusion, our present study identifies GDNF and NTN as autocrine/paracrine growth factors for CNS glia. One of the future challenges will be to define the role GDNF and NTN play during glial development and/or their adult function.

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