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Expression and regulation of GFRα3, a glial cell line-derived neurotrophic factor family receptor

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ABSTRACT **We report the identification of an additional member of the glial cell line-derived neurotrophic factor (GDNF) family receptor, termed GFRα3, that is homologous to the previously identified GDNF and neurturin ligand binding receptors GFRα1 and GFRα2. GFRα3 is 32% and 37% identical to GFRα1 and GFRα2, respectively. RNase protection assays show that whereas gfrα1 and gfrα2 are abundant in both developing and adult brain, gfrα3 is exclusively expressed during development. All receptors are widely present in both the developing and adult peripheral nervous system and in peripheral organs. For instance, *in situ* hybridization shows that the developing liver, stomach, intestine, kidney, and sympathetic chain, which all contain ret-expressing cells, transcribe unique complementary and overlapping patterns of most or all of the GDNF family receptors and ligands. In sensory neurons of the trigeminal ganglion gfrα2 and gfrα3 are expressed in different subpopulations of neurons, whereas gfrα1 is coexpressed in some gfrα2 and gfrα3-positive neurons. We find that the gfrα1 population of trigeminal neurons is absent in GDNF null mutant mice, suggesting that GDNF signals *in vivo* by interacting with GFRα1. Thus, our results show that there are at least three members in the GDNF family of ligand binding receptors and that these receptors may be crucial in conferring ligand specificity *in vivo*. The unique complementary and overlapping expression of gfrα3 implies distinct functions in the developing and adult mouse from that of GFRα1 and GFRα2.**

In the vertebrate nervous system, the survival of many neuronal populations is controlled by neurotrophic factors often produced by the targets of innervation. A very well-characterized family of neurotrophic factors is the neurotrophin (NT) family, which includes nerve growth factor, brain-derived neurotrophic factor, NT3, and NT4 (1). These neurotrophic factors display a remarkable complementary and cooperative action during development of the peripheral and central nervous systems. Many of the different functional classes of dorsal root, trigeminal, vestibular, and auditory ganglion neurons depend for their survival on specific NTs (2–14), and consistently different functional classes of neurons express different NT receptors (15, 16). NT3 also has been suggested to play a role for somatic sensory neurons before the period of programmed cell death affecting survival and differentiation (13, 14, 17–21), and many cultured neurons that initially require NT3 become dependent on another NT member at later developmental stages (22, 23). More recently a developmental switch in NT dependency has been shown to occur also *in vivo* in NT gene-targeted null mutant mice (24). The switch from NT3 dependency is paralleled by the down-regulation of the NT3 receptor, trkC (25).

Recently, a family of neurotrophic factors has been discovered, including the structurally related ligands glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) (26, 27). They are distant members to the transforming growth factor β family. GDNF is a potent survival factor for several populations of central neurons in culture and *in vivo*. GDNF protects lesioned adult substantia nigra dopaminergic neurons from death (26, 28–30) and rescues developing and lesioned adult spinal cord motor neurons (31–35). GDNF also promotes the survival and morphologic differentiation of cerebellar Purkinje cells and lesioned *locus coeruleus* noradrenergic neurons (36, 37). *In vitro*, GDNF promotes the survival of dissociated chicken parasympathetic, sympathetic, visceral, and cutaneous sensory neurons (38, 39) and rat sensory and sympathetic neurons (40). A neurotrophic role for GDNF in peripheral neurons recently has been directly demonstrated in GDNF null mutant mice (34, 35, 41). GDNF also has been shown to be involved during the inductive epithelial-mesenchymal interactions that accompany kidney organogenesis (34, 35, 41). Less is known about the functions of NTN. In culture, this factor has been shown to stimulate the survival of peripheral sensory and sympathetic neurons (27).

GDNF family ligands thus are secreted molecules that play fundamental roles during inductive events of organogenesis as well as for cell survival and differentiation in the nervous system. Last year components of the receptor system mediating the effects of GDNF and NTN were discovered. The glycosylphosphatidylinositol membrane-linked receptor subunit, GDNFRα/TrnR1, which will be referred to as GDNF family receptor α1 (GFRα1), binds GDNF. The complex GDNF-GFRα1 is required for subsequent Ret binding and activation (42–45).

Recently, a novel receptor TrnR2/NTNR-a/RETL1 (GFRα2) displaying close to 50% amino acid homology to GFRα1 was identified and characterized (39, 46–48). GDNF and NTN can activate Ret (49) in cultured cell lines by interacting with either GFRα1 or GFRα2. However, when present at low concentrations GDNF and NTN can display a preference for GFRα1 and GFRα2, respectively (39, 47).

We report the identification of a receptor called GFRα3 based on homology to GFRα1 and GFRα2. GFRα3 is 32% and 37% identical to GFRα1 and GFRα2, respectively. Our characterization of this receptor suggests that it play roles in peripheral tissues and in the nervous system that are distinct from those of GFRα1 and GFRα2.

Abbreviations: GDNF, glial cell line-derived neurotrophic factor; GFRα1, 2, or 3, GDNF family receptor 1, 2, or 3; NTN, neurturin; NT, neurotrophin; EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; E, embryonic day.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF036163).

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MATERIALS AND METHODS

Gfrα3 Cloning. GFRα3 was identified by a BLAST search in the dbEST sequence databank by using the protein sequence of GFRα1 as template (43). Two mouse expressed sequence tags (ESTs) were significantly different from both GFRα1 and GFRα2 (mj11d08 and mj08d05). These two cDNAs were obtained through the American Type Culture Collection and subcloned in pBSKS+. A restriction enzyme map was established, and short pieces of the cDNAs were subcloned and subjected to DNA sequence analysis. The deduced amino acid sequence showed that the complete ORF of GFRα3 was present in one of the EST clones. However, this clone lacked one nucleotide, causing a translational frame-shift at amino acid 69 in GFRα3. Reverse transcriptase–PCR therefore was used to clone a 850-bp cDNA fragment spanning this region of GFRα3 by using mouse whisker follicle and heart RNA as templates. Sequence analysis of such cDNA fragments revealed the presence of an additional nucleotide, a thymidine, at leucine 69 in GFRα3 in all cDNAs sequenced, suggesting a point mutation in the ATCC cDNA clone.

Probes. The rat GFRα1 and the mouse ret probes have been described previously (50). The rat GDNF, the mouse GFRα2, GFRα3, and NTN cDNA probes corresponding to nucleotides 380–800 (26), 805-1215 (46), 601–910 (this paper), and 613–963 (27), respectively, was prepared as follows. cDNAs were isolated by reverse transcriptase–PCR using total RNA as template. The first-strand synthesis was primed with oligo(dT) primers (Pharmacia, Uppsala) and avian myeloblastosis virus reverse transcriptase (Promega). The cDNA fragments then were amplified by PCR using *Taq* polymerase and primers within the reported mouse ORFs. In the case of NTN, the Expand Long Template PCR System (Boerhinger Mannheim) was used instead of *Taq* polymerase. The cDNAs were cloned into pBluescript and subjected to sequence analysis. All cRNA probes were synthesized from linearized plasmids as previously described (50). The rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cRNA probe (51) used here as an internal control resulted in a 160-nt-long fragment.

RNase Protection Assay. Pregnant BALB/c mice were killed by cervical dislocalization at different developmental time points, and the embryonic brains were dissected and frozen at −70°C until used. Adult BALB/c mice were dissected, and different parts of the brain and peripheral organs were collected. Total RNA were extracted by using the LiCl-Urea procedure described by Auffray and Rougeon (52). RNase protection assays were performed by using 10 μg of total RNA as previously described (50).

***In Situ* Hybridization Procedure.** For *in situ* hybridization, tissues of time-staged embryos or postnatal mice were positioned on a metal block, frozen, and sectioned transversely (14 μm) on a Leitz cryostat. All sections were thaw-mounted onto slides pretreated with 3-aminopropyl triethoxysilane (Sigma) and kept frozen until hybridization. Before use, the sections were fixed in 4% paraformaldehyde for 15 min and rinsed twice in PBS (pH 7.5) and twice in distilled water. A previously described protocol for nonradioactive digoxygenin UTP-ribonucleotide *in situ* hybridization then was used (www.cco.caltech.edu/≈mercer/htmls/Big_in_situ.html).

The labeled neurons in the trigeminal ganglion were captured by using a 20× objective on every eighth section (control mice $n = 6$, gdnf$^{-/-}$ mice $n = 5$), and the images were processed by using the IP-lab spectrum software (Signal Analytics, Vienna, VA). The signal-to-noise ratio of the *in situ* hybridization was very high (see figures), and the threshold for the identification of labeled cells therefore could be set high. The total number of trigeminal neurons and receptor expressing neurons were determined as previously described (13).

Genotyping of GDNF Mutant Mice. Heterozygous GDNF mutant mice were bred, and their offspring were collected at birth and used for the experiments. All of the neonatal mice were genotyped for the wild-type and gdnf mutant alleles by PCR according to Pichel *et al.* (41).

RESULTS

GFRα3 Is a GDNF Family Receptor Homologue. GFRα3 was identified by a BLAST search in the dbEST sequence databank by using the protein sequence of GFRα1 as template (43, 44). A large number of ESTs with partial but significant homology to GFRα1 were identified. Aligning the sequences to GFRα2 revealed that the majority of the cDNA clones encoded this receptor. Two mouse ESTs were significantly different from both GFRα1 and GFRα2 (mj11d08 and mj08d05). These two cDNAs were obtained through the American Type Culture Collection, subjected to restriction enzyme mapping, subcloned, and DNA-sequenced. The sequence analysis revealed that the two ESTs represented cDNAs of varying length encoding the same protein. One of the cDNAs was full length and contained a deduced protein of 397 amino acids (Fig. 1, see *Materials and Methods* for details).

The sequence around the first ATG of GFRα3 provided a strong context for initiation (53) with GCC in positions −3, −2, and −1 and a G in position +1. Aligning GFRα3 to GFRα1 and GFRα2 revealed 32% and 37% amino acid identity, respectively. Similar to GFRα1 and GFRα2, GFRα3 contains a possible signal sequence, three potential N-glycosylation sites (amino acids 92–95, 145–148, and 306–309) and a putative glycosylphosphatidylinositol-linked hydrophobic C terminus. Furthermore, all 28 cysteins of GFRα3 were conserved in GFRα1 and GFRα2. The result that GFRα3 displays a lower homology to GFRα1 and GFRα2 than GFRα1 and GFRα2 does suggests that GFRα3 is a more distant member in this family of receptors.

GFRα3 Is Expressed Only During Early Stages of Neurogenesis in the Central Nervous System Whereas GDNF, NTN, and Their Receptors are Abundant Throughout Development. cDNA fragments of appropriate sizes to be transcribed and used as probes for RNase protection assays were generated for GDNF family ligands and receptors. All RNase protection assays were adjusted to the GAPDH internal control for equal loading. Although presented in arbitrary units the relative

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gfra1   MFLATLYFALPLLDLLM              SAEVSGGD          RLDCVKAS
gfra2   MILANAFCLFFFLDETLR             SLASPSSPQGSELHGWRPQVDCVRANE
gfra3   MGLSLEPRPPL LMILLLVLSLWLPLGAG NSLATENRFVN          SCTQARK

gfra1   D QCLKEQSCSTKYRTLRQCVAGKETNFSLTSGLEAKD    ECRSAMEALKQKSLYN
gfra2   FTLCAAESNCSSRYRTLRQCLAGRDRN     TMLANK    ECQAALEVLQESPLYD
gfra3   K  CEANPACKAAYQHLGSCTSSLSRP     LPLEESAMSADCLEAAEQLRNSSLID
                                                    ****

gfra1   CRCKRGMKKEKN  CLRIYWSMYQSL QGNDLLEDSPYE PVNSRLSDIFRAVPFISDV
gfra2   CRCKRGMKKELQFTCLQIYWSIHLGLTEGEEFYEASPYE PVTSRLSDIFRLASIFSGT
gfra3   CRCHRRMKH  QATCLDIYWTVHPARSLGDYELDVSPYEDTVTSKPWKM          NL
                                                                   **

gfra1   FQQVEHISKGNNCLD  AAKACNLDDTCKKYRSAYITPCTTSMS NEVCNRRKCHKALR
gfra2   GADPVVSAKSNHCLDFTAAKACNLNDNCKKLRSSYISICNREISPTERCNRRKCHKALR
gfra3   SKLNMLKPDSDLCLKF  AMLCTLHDKCDRLRKAYGEACS      GIRCQRHLCLAQLR
        **

gfra1   QFFDKVPAKHSYGMLFC  SCR  DIACTERRRQTIVPVCSYEERERPNCLSLQDSCKT
gfra2   QFFDRVPSEYTYRMLFCFTSCQ  DQACAERRRQTILPSCSYEDKEKPNCLDLRSLCRT
gfra3   SFFEKAAESHAQGLLLCP  CPPEDAGCGERRRNTIAPSCALP SVTPNCLDLRSFCRA

gfra1   NYICRSRLADFFTNCQPESR  SVSNCLKENYADCLLAYSGLIGTVMTPNYVVDSSSL
gfra2   DHLCRSRLADFHANCRASYRTFTITSCPADNYQACLGSYAGMIGFDMTPNYVVDSNPTGI
gfra3   DPLCRSRLMDFQTHCHPM   DILGTCATEQSR CLRAYLGLIGTAMTPNFI SKVNTT
                                                              ***

gfra1   SVAPWCDCSNSGNDLEDCLKFLNF  FKDNTCLKNAIQAFGNGSDVTMWQPAPPVQTTT
gfra2   VVSPWCNCRGSGNMEEECEKFLKFTDFTENPCLRNAIQAFGNGTDVNMSPKGPTFSATQ
gfra3    VALSCTCRGSGNLQDECEQLER  SFSQNPCLVEAIAA        KMRFHRQLFSQD
         *

gfra1   ATTTTAFRVKNKPLGPAGSENEIPTHVL  PPCANLQAQKLKSNVSGSTHLCLSDSDFG
gfra2   APRVEKTPSLPDDLSDSTS   LGTSVIFTTTCTSIQEQGLKANNSKELSMCFTELTTN
gfra3     WADSTFSVVQQQNSNPA

gfra1   KDGLAGASSHITTKSMAAPPSCSLSS LPVLM LTALAALLSVSLAETS
gfra2   ISPGSKKVIKLYSGSCRARLSTALTA LPLLFTMVTLA
gfra3                      LR LQPRLPILSFSI LPLILLQTLW
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FIG. 1. Deduced amino acid sequence of mouse GFRα3 aligned to mouse GFRα1 and mouse GFRα2. Identical residues are in bold. The N-terminal putative signal sequence and C-terminal hydrophobic domain are underlined. The three potential N-glycolysation sites are indicated by *.

levels were obtained from a PhosphorImager and standardized such that the signals should be comparable between probes and experiments. In each autoradiogram of Figs. 2–4, the internal GAPDH also is presented as a reference for the amount of RNA used.

RNase protection assay of embryonic and adult total brain tissue revealed gfrα3 transcripts as early as embryonic day (E)12 and E15. Expression of gfrα3 was down-regulated to barely detectable levels by E18 and postnatal day (P) 1, and was below the detection limit at P3. The levels remained below the detection limit throughout the rest of postnatal life (Fig. 2). In contrast to gfrα3, gfrα1 and gfrα2 expression levels peaked around E15 after which they progressively declined to lower but significant adult levels (Fig. 2).

GDNF Family Ligands and Receptors in the Adult Brain. The adult mouse brain was dissected and RNA prepared from 10 brain regions for the RNase protection assay. Whereas gfrα1 and gfrα2 transcripts were detected in all brain regions, gfrα3 transcripts were not detectable (Fig. 3). GDNF transcripts, like the abundant transcripts of gfrα1 and gfrα2, were present in most of the analyzed brain regions, and NTN transcripts were detected in all of them. Ret mRNA was abundant in the olfactory bulb, septum, thalamus, hypothalamus, colliculi, cerebellum, and brain stem.

GFRα3 Is Widely Expressed in Developing and Adult Peripheral Organs. In the adult mouse, gfrα3 messenger was detected in peripheral organs and ganglia. Its expression was remarkably closely linked to the one of ret (Fig. 4). Trigeminal ganglion, pituitary gland, thymus, lung, and duodenum expressed gfrα3 in proportions that resemble ret expression. Heart, kidney, muscle, and liver that contained no or low levels of ret also expressed little or no gfrα3. Gfrα1 and gfrα2 were more widely expressed and were present in most peripheral organs. The only organ expressing one GDNF family receptor member was the spleen where exclusively gfrα2 was present.

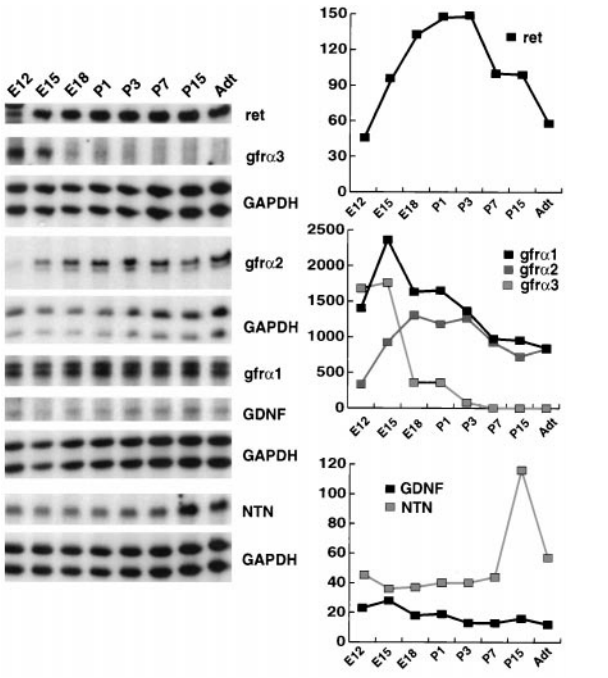

FIG. 2. RNase protection assay for the detection of ret, GDNF, NTN, and gfrα receptors during brain development. The brain was collected at different time points before or after birth. Total RNAs were extracted and submitted to RNase protection assay by using the indicated probes. Autoradiograms are presented at *Left*. (*Right*) The graphs represent the average of radioactivity value detected on a PhosphorImager expressed in arbitrary units and displayed as [(value X/value GAPDH) $\times$ 1,000]. To be able to show the grfα3 regulation in the same graph as the two other members of the family, values for gfrα3 was multiplied by 40. P, postnatal day; Adt, adult.

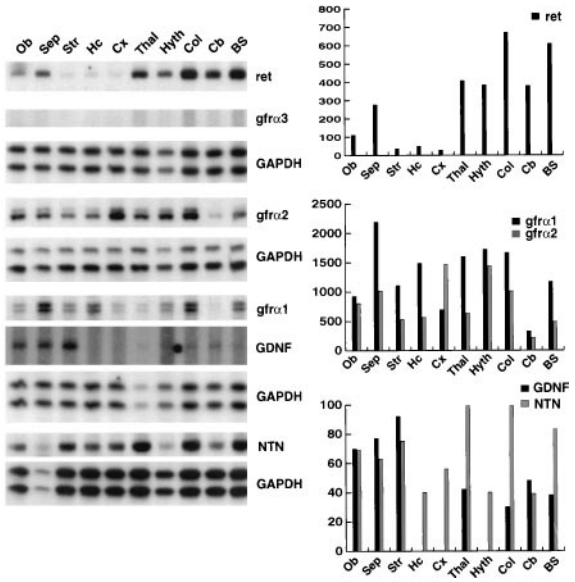

FIG. 3. Expression of gfrα receptors, ret, GDNF, and NTN in different brain regions of the adult mouse. Different brain regions were dissected from BALB/c mice, and total RNAs were extracted as described in *Materials and Methods*. (*Left*) Autoradiograms of RNase protection assays. (*Right*) Graphs represent the average of the PhosphorImager values expressed in an arbitrary unit [(value X/value GAPDH) $\times$ 10,000]. Ob, olphactory bulb; Sep, septum; Str, striatum; Hc, hippocampus; Cx, cortex; Thal, thalamus; Hyth, hypothalamus, Col, colliculi; Cb, cerebellum; BS, brain stem.

We used nonradioactive *in situ* hybridization to identify cells expressing GDNF family receptors and ligands in mouse embryonic tissues. At E13, GDNF and NTN were expressed in many organs such as the liver, mesenchyme of the kidney, mucosal epithelium of the intestine, stomach, and in the

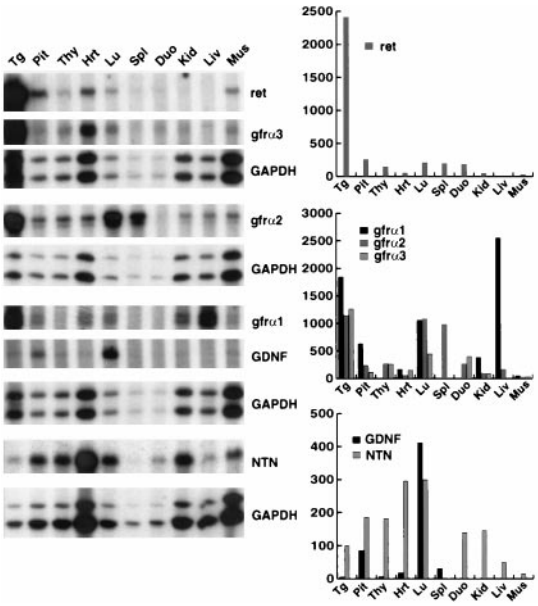

FIG. 4. Expression of gfrα receptors, ret and GDNF and NTN in peripheral organs and neurons. Peripheral organs and the trigeminal ganglion of BALB/c mice were dissected, and total RNAs were extracted. (*Left*) Autoradiograms of RNAs protection assays. (*Right*) Graphs represent the average of the PhosphorImager values expressed in an arbitrary unit [(value X/value GAPDH) $\times$ 10,000]. Tg, trigeminal ganglia; Pit, pituitary gland; Thy, thyroid; Hrt, heart; Lu, lung; Spl, spleen, Duo, duodenum; Kid, kidney, Liv, liver; Mus, muscle.

sympathetic chain (the latter only NTN, Fig. 5). Interestingly, gfrα3 mRNA expression closely resembled that of GDNF and NTN being present in the mesenchyme of kidney, mucosal epithelium of the intestine, and sympathetic chain. In contrast, ret expression was localized to epithelial buds of the kidney and scattered cells in the smooth muscle layers of the intestine and stomach, which also often contained gfrα1 mRNA (Fig. 5). At E18 GDNF and NTN remained in the mucosal lining of the stomach and intestine, which at this stage also contained ret, and all GDNF family receptors (data not shown). NTN, GDNF, and all GDNF family receptors also were present in developing glomeruli of the kidney.

GFRα1–3 Transcripts Are Individually or Jointly Expressed in Distinct Subpopulations of Trigeminal Ganglion Neurons. Abundant levels of ret and gfrα1–3 transcripts were detected in the adult trigeminal ganglion by RNase protection assay (Fig. 4). By using *in situ* hybridization to identify the cells expressing gfrα1–3 and ret in the trigeminal ganglion, all GDNF family receptors were clearly detected in neurons of the newborn (Fig. 6) and adult trigeminal ganglion. Already during the initial examination of the sections it was evident that the receptors were expressed in a remarkably specific pattern within the ganglion. Gfrα1-expressing cells were scattered throughout the ganglion, whereas gfrα2-positive cells were concentrated in the ventral and gfrα3-positive cells in the dorsal aspect of the ganglion. Simultaneous detection of gfrα2 and gfrα3 transcription led to a pattern of labeled neurons resembling ret.

The total number of trigeminal ganglion neurons was counted in cresyl violet-stained sections, and the number of gfrα1–3 and ret-labeled neurons was measured. The percentage of trigeminal ganglion neurons expressing each receptor was calculated. Gfrα1 and gfrα2 both were expressed in approximately 10% of the trigeminal ganglion neurons. Almost 20% of the neurons were positive for gfrα3 mRNA expression (Fig. 6). The complementary pattern of gfrα1–3 expression and the results showing that as much as 37% of the trigeminal ganglion neurons express ret mRNA (Fig. 6) suggest

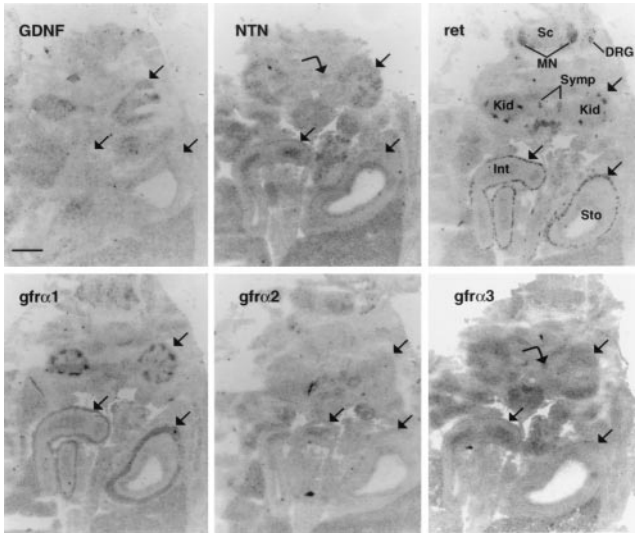

FIG. 5. Nonradioactive *in situ* hybridization of adjacent coronal sections from an E13 mouse embryo hybridized for the detection of GDNF, NTN, ret, gfrα1, gfrα2, and gfrα3 transcripts as indicated. Straight arrows indicate the kidney, intestine, and stomach, curved arrows the sympathetic chain. Note labeling of GDNF, NTN, and gfrα3 in mesenchyme of kidney, and gfrα1 and ret in the kidney epithelial buds. Also note the complementary and overlapping expression of ligands and receptors in the intestine, kidney, and stomach. DRG, dorsal root ganglion; Int, intestine; Kid, kidney; MN, motor neuron column; Sc, spinal cord; sto, stomach; Symp, sympathetic chain. (Scale bar is 440 μm.)

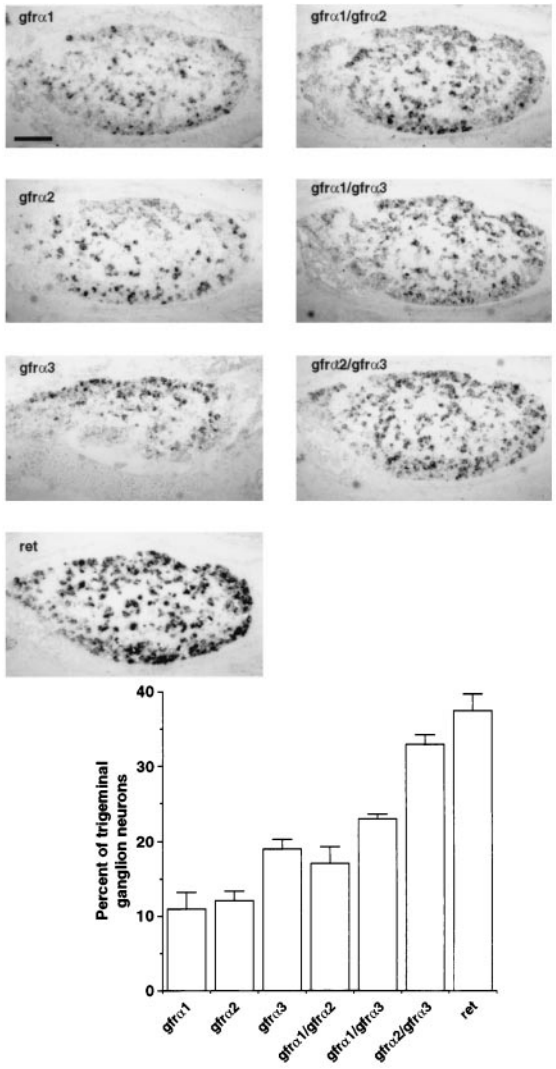

FIG. 6. Nonradioactive *in situ* hybridization for gfrα receptors and ret in the neonatal mouse trigeminal ganglion. Micrographs of the trigeminal ganglion are shown. Sections from the trigeminal ganglion were hybridized with either a single probe or with cocktails containing two different probes as indicated. Note that gfrα2 and gfrα3 are preferentially expressed in neurons of the ventral and dorsal aspects of the trigeminal ganglion, respectively. The numbers of labeled neurons with each probe and combination of probes was quantified, and the total number of neurons in the trigeminal ganglion was counted on adjacent cresyl violet-stained sections. The percent of trigeminal ganglion neurons labeled for each receptor(s) thereafter was calculated and plotted in the graph ($n = 4$, Student's t test; $P < 0.05$ gfrα3-gfrα1/gfrα3, $P < 0.001$ gfrα3-gfrα2/gfrα3). (Scale bar is 170 μm.)

that GDNF family receptors could be expressed in different subpopulations of ganglion neurons.

To investigate this possibility, we hybridized sections of the trigeminal ganglion with cocktails containing mixes of two riboprobes. There was an additive increase of labeled neurons when a mixture of gfrα2/gfrα3 probes were used (33% of the neurons) compared with individual probe hybridization (12% and 18%, respectively), showing that these receptors are largely expressed in distinct subpopulations of trigeminal ganglion neurons. A significant increase in the number of labeled neurons also was seen when the gfrα1/gfrα2 mixture of probes was used compared with single probes (Fig. 6), whereas only a small increase in the number of labeled neurons was seen in sections hybridized with the gfrα1/gfrα3 mix of probes. Thus, many of the gfrα1 cells also express gfrα3 and

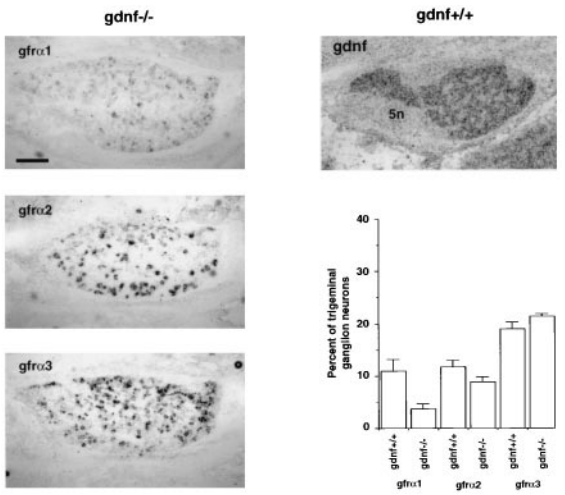

FIG. 7. Detection of gfrα receptors in GDNF null mutant mice. Neonatal wild-type mice were hybridized with the GDNF probe and neonatal $GDNF^{-/-}$ and $GDNF^{+/+}$ mice with the gfrα1, gfrα2, or gfrα3 probe as indicated. Note expression of GDNF in trigeminal ganglion neurons. Also note the marked loss of gfrα1-positive neurons, but the absence of change in gfrα2 or gfrα3-expressing neurons. The graph represents a quantification of the percent of the trigeminal ganglion neurons expressing the different gfrα receptors. The reduction in gfrα1 expressing neurons in $GDNF^{-/-}$ mice compared with $GDNF^{+/+}$ mice was statistically significant ($n = 4$, Student's t test, $P < 0.01$). 5n, trigeminal nerve. (Scale bar is 170 μm.)

some coexpress gfrα2. However, there are few or no neurons coexpressing gfrα2 and gfrα3.

GFRα1 Is a GDNF Receptor *in Vivo*. GDNF and NTN receptor interactions have been studied previously in cell culture. We sought to determine whether GDNF has a preference for any of the GDNF family receptors *in vivo*, including the GFRα3 receptor. We used for this experiment GDNF null mutant mice ($gdnf^{-/-}$ mice), asking whether the absence of GDNF *in vivo* affects neurons expressing a specific GDNF family receptor member. However, we first examined the extent of GDNF transcription in the trigeminal ganglion. *In situ* hybridization revealed that most neurons in the trigeminal ganglion of normal mice expressed GDNF transcripts at E16, E18 as well as at birth (Fig. 7). When we next examined the expression of GDNF family receptors in the GDNF null mutant mice we found that gfrα1-positive cells were markedly reduced in the trigeminal ganglion, whereas no change in the number of gfrα2 or gfrα3 expressing cells was observed (Fig. 7). Quantification confirmed that the loss of gfrα1-positive cells in the gdnf compared with $gdnf^{-/-}$ mice was significant. Thus, these results suggest that GFRα1 is the preferred receptor for GDNF *in vivo* in the trigeminal ganglion.

DISCUSSION

We describe the identification and characterization of a GDNF family receptor homologue, termed GFRα3. This receptor is 32% and 37% homologous to GFRα1 and GFRα2, respectively. The specificities of ligand interaction of the latter two have been established *in vitro*. In the absence of Ret, GFRα1 binds only to GDNF whereas GFRα2 binds only to NTN (46, 47). In the presence of Ret GFRα2 also can bind GDNF if it is present at sufficient concentrations (48) and sympathetic neurons expressing GFRα2 survive in response to GDNF, although NTN rescues more neurons (39). Furthermore, GFRα2 distinguishes between NTN and GDNF by inducing Ret phosphorylation at lower concentrations of NTN than GDNF ligand in cultured fibroblasts. However, GFRα1 mediates Ret phosphorylation at similar concentrations of GDNF and NTN (46). Most of the above studies were performed either by measuring physical interactions in cell-free systems or by receptor binding and phosphorylation on cultured cell lines. Interestingly, when analyzed in a neuronal survival assay *in vitro* GFRα1 and GFRα2 display higher specificity toward low doses of GDNF and NTN, respectively, compared with studies on fibroblast cell lines (39). Because the specificity of tyrosine kinase receptors sometimes have been shown to be different *in vivo* than under culture conditions (54) we sought to determine whether GFRα2 and GFRα3 mediate GDNF signaling *in vivo* in the trigeminal ganglion and used for this experiment GDNF null mutant mice (41). Our results suggest that at the level of GDNF present in the trigeminal ganglion during normal development only GFRα1 expressing neurons are affected. We also have found that NTN is expressed in developing trigeminal ganglion neurons (unpublished results). The absence of gfrα1 cells therefore also suggests that NTN is unable to compensate for the loss of GDNF in the null mutant mice. We conclude that GDNF could be the preferred ligand for GFRα1 *in vivo* and that GDNF family receptors may bestow higher specificity *in vivo* than under culture conditions.

We found that NTN was expressed in the adult duodenum, kidney, and liver, but GDNF and gfrα1 had been downregulated. The only GDNF family receptor present in all of these adult tissues was GFRα2. Our findings therefore are consistent with previous results that have shown that NTN could be the preferred GFRα2 ligand and, furthermore, suggest a role for NTN in these organs.

What is the ligand interacting with the GFRα3 receptor? Our *in vivo* results suggest that it may not be GDNF in the developing trigeminal ganglion. Surprisingly, we found that most peripheral tissues express more than one GDNF family receptor member. Similar results also were obtained for a subpopulation of neurons in the trigeminal ganglion, which coexpressed GFRα2 and GFRα3 with GFRα1. GDNF is active as a homodimer and is believed to interact with dimers of GDNF family receptors. The results showing that many tissues and cells express more than one GDNF family receptor therefore may not necessarily suggest that they depend on more than one GDNF family ligand. Instead, it also opens up the possibility that heterodimer combinations of GFRα1, GFRα2, and GFRα3 generate new receptors with distinct functions and pharmacological binding profiles for GDNF and NTN. In such a case, the role of a GDNF family receptor could exclusively be to modulate ligand binding to other receptor members. However, because most gfrα3-positive trigeminal ganglion neurons did not express gfrα1 or gfrα2, GFRα3 may at least in some instances play a role independent from GFRα1 and GFRα2. Data recently reported (55) indicate the existence of a third member in the GDNF family of ligands, termed persephin, which could be a GFRα3 receptor ligand.

Our results suggest that GDNF family receptors display ligand preference *in vivo* and that GFRα3 is a receptor with distinct roles from GFRα1 and GFRα2. The NTs have been shown to often support different functional classes of peripheral neurons. They do so by interacting with their receptors, which are restrictedly expressed in functionally distinct populations of sensory neurons (15, 16). The remarkable subpopulation-specific expression of gfrα1, gfrα2, and gfrα3 in the trigeminal ganglion shows that these receptors could play similar roles for GDNF family ligands during nervous system development.

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