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Multiple GPI-Anchored Receptors Control GDNF-Dependent and Independent Activation of the c-Ret Receptor Tyrosine Kinase[1]

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Glial cell line-derived neurotrophic factor (GDNF) mediates neuronal survival through a receptor complex composed of the c-*Ret* proto-oncogene and GFRα-1, a member of a family of GPI-anchored receptors. The extent of cross-talk between GDNF and GFRα receptors and its possible significance for c-Ret activation is presently unclear. Using chemical crosslinking we demonstrate here a specific interaction between GDNF and GFRα-2 expressed in COS cells, albeit of a lower affinity than the one between GDNF and GFRα-1. In addition, GFRα-2 mediated crosslinking of GDNF to c-Ret as well as ligand-dependent stimulation of c-Ret tyrosine phosphorylation. We also describe the isolation of a novel, more divergent member of the GFRα family, GFRα-3, which did not bind GDNF directly, but was able to mediate crosslinking of GDNF to c-Ret when both receptors were coexpressed in COS cells. Thus, all three GFRα receptors mediate GDNF binding to c-Ret with efficiencies GFRα-1>GFRα-2 >GFRα-3. c-Ret showed high levels of constitutive tyrosine autophosphorylation upon overexpression in COS cells, which was inhibited in a dose-dependent manner by coexpression with any of the GFRα receptors, suggesting that GFRαs may also provide a gain control mechanism to increase the signal-to-noise ratio of the response to ligand. GFRα-2 showed a dynamic pattern of expression in rat brain, distinct from that of GFRα-1, characterized by high expression in cortex, basal forebrain, and specific layers of the olfactory bulb, and low or no expression in substantia nigra, cerebellum, and motor nuclei. GFRα-2, but not GFRα-3 mRNA expression was highly induced in several nuclei after stimulation with kainic acid. In contrast to GFRα-1 and GFRα-2, GFRα-3 expression in postnatal and adult brain was highly restricted. Developmentally regulated expression of GFRα-3 was, however, detected in several peripheral organs and ganglia. Together, these results indicate complementary roles for GFRα receptors in the regulation of c-Ret activity and the maintenance of distinct neuronal circuits in the central and peripheral nervous systems.

INTRODUCTION

The *Ret* gene was discovered as an oncogene activated by rearrangement with a foreign sequence in a fibroblast focus-forming assay (Takahashi and Cooper, 1987). The multiple endocrine neoplasia (MEN) type 2 syndromes and Hirschsprung disease have been associated with germ-line mutations of the c-*Ret* proto-oncogene (Edery *et al.,* 1994; Hofstra *et al.,* 1994; Mulligan *et al.,* 1993; Romeo *et al.,* 1994). c-*Ret* encodes a receptor tyrosine kinase that plays a crucial role in kidney morphogenesis and in the survival and differentiation of several subpopulations of neurons in the peripheral and central nervous systems (Durbec *et al.,* 1996; Lo and Anderson, 1995; Schuchardt *et al.,* 1994). A major step toward understanding the biological actions of c-Ret was the recent identification of its ligand glial cell line-derived neurotrophic factor (GDNF) (Durbec *et al.,* 1996; Trupp *et al.,* 1996; Vega *et al.,* 1996; Worby *et al.,* 1996). GDNF is a polypeptide structurally related to members of the transforming growth factor-β (TGF-β) superfamily that was purified from the conditioned medium of a glial cell line on the basis of its ability to stimulate dopamine uptake in primary cultures of embryonic rat ventral midbrain neurons (Lin *et al.,* 1993). GDNF promotes the

[1] The GenBank accession numbers for the rat GFRα-2 and mouse GFRα-3 sequences reported in this manuscript are AF005226 and AF020305, respectively.

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survival and phenotype of several populations of peripheral and central neurons, including sympathetic, sensory, and motor neurons (Buj-Bello *et al.*, 1995; Henderson *et al.*, 1994; Oppenheim *et al.*, 1995; Trupp *et al.*, 1995; Yan *et al.*, 1995), as well as brain dopaminergic, noradrenergic, and cholinergic neurons (Arenas *et al.*, 1995; Beck *et al.*, 1996; Tomac *et al.*, 1995; Williams *et al.*, 1996). c-Ret is part of a multicomponent receptor complex for GDNF which includes a ligand binding subunit termed GDNF receptor-α (herein called GFRα-1 (GFRα Nomenclature Committee, 1997)), a glycosylphosphatidyl inositol (GPI)-anchored protein that is required for the physical association of GDNF with c-Ret and for ligand-induced activation of the c-Ret tyrosine kinase (Jing *et al.*, 1996; Treanor *et al.*, 1996). Importantly, the effects of GDNF and GFRα-1 on the biological activities of the various c-Ret mutants found in human diseases are completely unknown.

GDNF, c-Ret, and GFRα-1 show complementary and overlapping patterns of mRNA expression in the rat central nervous system (Trupp *et al.*, 1997). The fact that several regions in the adult rat brain appear to express only one of these two GDNF receptors prompted us to investigate the existence of additional receptor components. During the completion of this study, a receptor structurally related to GFRα-1 (TrnR-2 / NTNR-α / RETL-2 / GDNFR-β, herein called GFRα-2) was described as capable of mediating activation of c-Ret by a GDNF-related polypeptide, neurturin (NTN) (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Klein *et al.*, 1997; Sanicola *et al.*, 1997; Suvanto *et al.*, 1997). These studies, however, were in disagreement about the ability of this receptor to bind to and mediate the biological actions of GDNF. Here we describe the identification of a third member of the GFRα receptor family and present biochemical evidence for cross-talk between GDNF and members of this receptor family, and for a ligand-independent interaction between GFRα receptors and c-Ret at the cell membrane. We also report on the mRNA distribution of GFRα-2 and GFRα-3, which suggests broader neuronal as well as nonneuronal actions for GDNF family ligands.

RESULTS

Molecular Cloning of Receptors Structurally Related to GFRα-1

A BLAST search of the Genbank Expressed Sequence Tag (EST) database for sequences similar to rat GFRα-1 (Jing *et al.*, 1996) resulted in the identification of several ESTs that were related but not identical to this receptor. Primers based on the sequence of the human EST H12981 were used for PCR amplification of first strand human brain cDNA. The amplified fragment was used to screen a postnatal day 7 (P7) rat brain cDNA library from which several hybridizing clones were isolated and purified. Additional screening and PCR led to the identification of an open reading frame coding for a putative 464 amino acid residue polypeptide (herein called GFRα-2) showing 63% similarity and 49% identity to rat GFRα-1 (Fig. 1A). The predicted protein sequence of rat GFRα-2 was 97 and 94% identical to mouse and human TrnR-2 / NTNR-α / RETL-2 / GDNFR-β, a recently isolated receptor found to mediate activation of c-Ret by GDNF and related ligands (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Klein *et al.*, 1997; Sanicola *et al.*, 1997; Suvanto *et al.*, 1997). Two mouse ESTs (AA049894 and AA050083) showing amino acid similarity to GFRα-1, but distinct from GFRα-2, were also found. The sequence of EST AA050083 extended more upstream and appeared to represent a full-length cDNA, except that it contained a frame-shift in the upstream portion of the clone. Extension of the 5′ end of clone AA049894 by RACE analysis confirmed that a T nucleotide was missing at position 251 of clone AA050083. A full-length cDNA was then assembled joining the RACE product to the 5′ end of clone AA049894. The resulting DNA fragment encoded a putative 397 amino acid residue polypeptide (herein called GFRα-3) showing 50% similarity and 35% identity to GFRα-1 (Fig. 1A). GFRα-3 is a more divergent member of the GFRα receptor family (Fig. 1B), and these structural differences also extended to its function and expression patterns (see below). The predicted protein sequence of both GFRα-2 and GFRα-3 included a putative signal peptide sequence at the N-terminus and a hydrophobic C-terminal motif reminiscent of the GPI-anchor signal sequence of GFRα-1 (Fig. 1A). Six potential N-glycosylation sites were found in the predicted protein sequence of GFRα-2, compared to only three sites in the GFRα-1 and GFRα-3 sequences (Fig. 1A). In agreement with this indication, GFRα-2 expressed in COS cells had a higher apparent molecular weight than GFRα-1 in SDS / PAGE gels (data not shown) despite being a few residues shorter, presumably due to differences in glycosylation.

GFRα-2 and GFRα-3 Mediate GDNF Binding to c-Ret

A GFRα-2 expression plasmid was transfected into COS cells either alone or together with a c-Ret expression construct. A parallel set of transfections were performed with a GFRα-1 expression plasmid. Transfected COS cells were used in cross-linking assays to assess the ability of GFRα-2 to bind GDNF and to allow cross-linking of GDNF to c-Ret. Using ethyl-dimethyl-aminopropyl carbodiimide (EDAC) as crosslinking

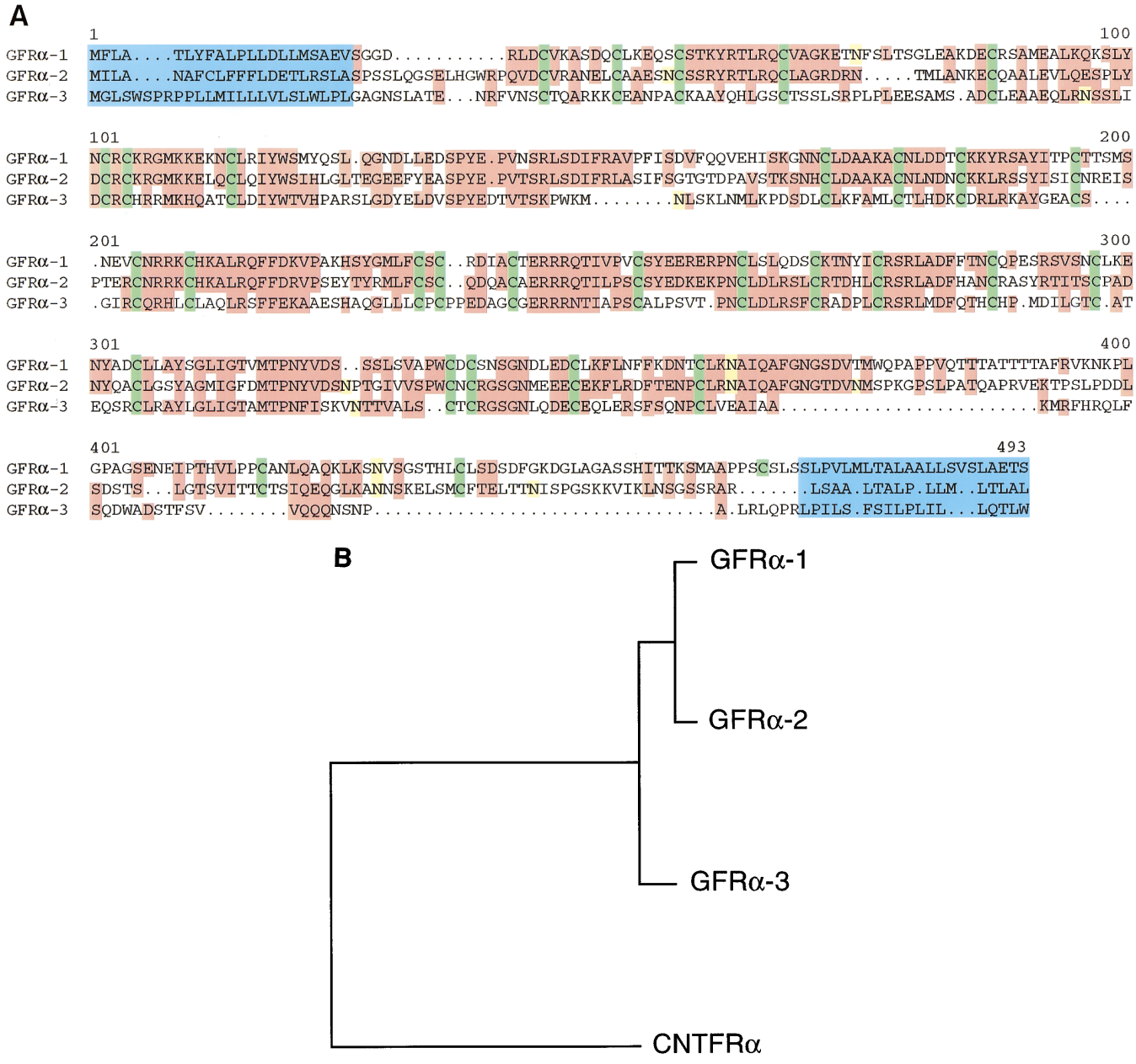


FIG. 1. The family of GFRα receptors. (A) Alignment of the predicted amino acid sequences of GFRα-1, GFRα-2, and GFRα-3 generated with PILEUP (Genetics Computer Group, 1994). Putative signal sequences and GPI anchor signals are boxed in blue at the N- and C-termini of the proteins, respectively. Potential N-glycosylation sites are boxed in yellow. Amino acid identities and similarities are boxed in red. (B) Phylogenetic tree of the GFRα family generated with GROWTREE (Genetics Computer Group, 1994). Rat ciliary neurotrophic factor receptor-α (CNTFR-α) was used as outgroup.

agent, a complex between ^{125}I-GDNF and GFRα-2 could be seen in transfected COS cells (Fig. 2A, lane 5). Formation of this complex could be prevented by excess unlabeled GDNF, indicating that it represented specific binding of GDNF to GFRα-2 (Fig. 2A, lane 6). Similar to GFRα-2 itself, this complex was of a somewhat higher molecular weight than the one between GDNF and GFRα-1 (Fig. 2A, compare lanes 3 and 5). Crosslinking of GDNF to GFRα-2 was, however, less efficient than to GFRα-1 (Figs. 2A and 2B). c-Ret was unable to bind GDNF unless coexpressed together with GFRα receptor subunits (Fig. 2A, lane 11). Importantly, GFRα-2 was at least as efficient as GFRα-1 in mediating affinity labeling of c-Ret by ^{125}I-GDNF (Fig. 2A, lanes 7 to 10).

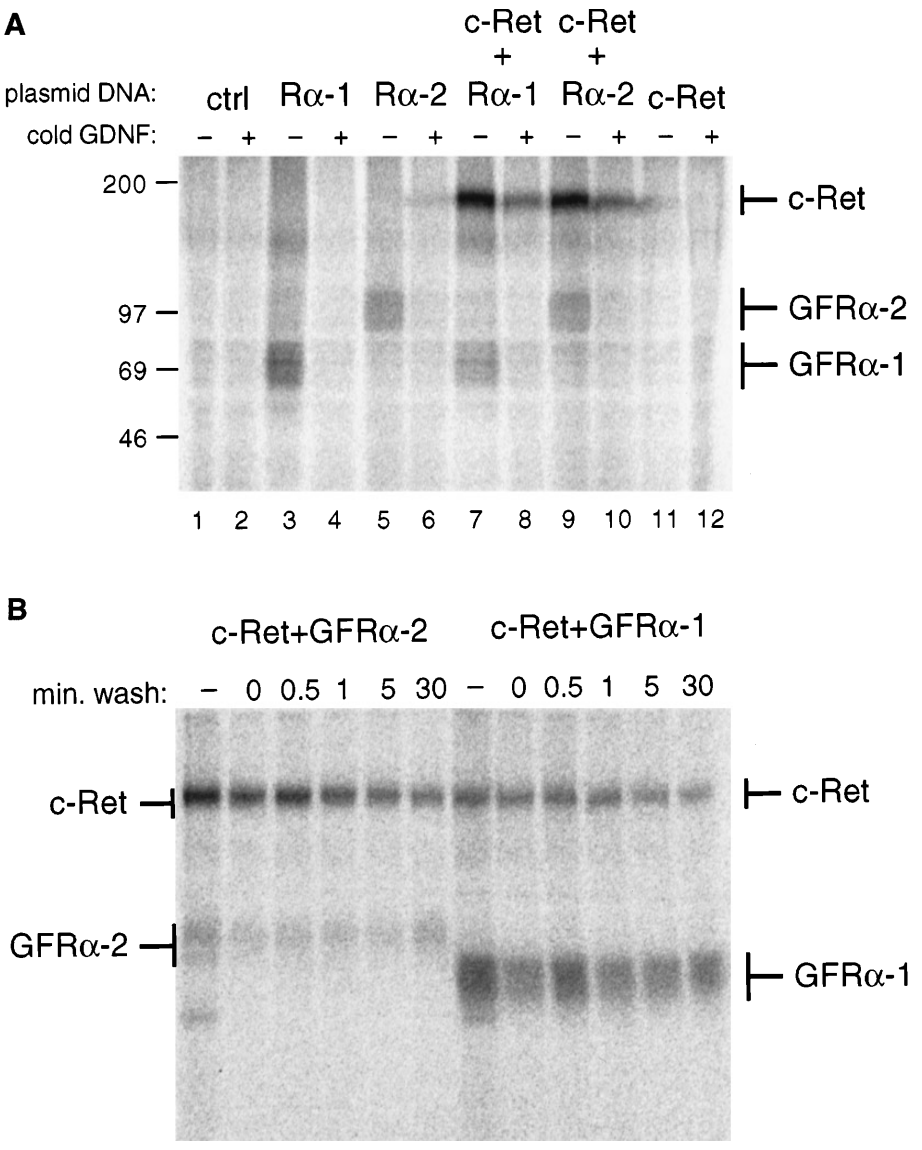


FIG. 2. GDNF binding to GFRα-1, GFRα-2, and c-Ret in transfected COS cells. (A) ^{125}I-GDNF was allowed to bind to COS cells transiently transfected with the indicated plasmid DNA constructs in the presence (+) or absence (−) of an excess of unlabeled (cold) GDNF as indicated. Complexes between ^{125}I-GDNF and c-Ret, GFRα-1 or GFRα-2, respectively, are indicated. No affinity-labeled complexes were seen in COS cells transfected with control (ctrl, lanes 1 and 2) or c-Ret (lanes 11 and 12) expressing plasmids. Specific complexes of approximately 70K and 90K were seen in COS cells expressing GFRα-1 (lanes 3 and 4) or GFRα-2 (lanes 5 and 6), respectively. Addition of both GPI-linked receptors allowed equally efficient labeling of c-Ret with ^{125}I-GDNF (compare lanes 7 and 9). In the experiment shown, residual binding of ^{125}I-GDNF to c-Ret could still be seen in the presence of excess cold GDNF (lanes 8 and 10). (B) Dissociation of ^{125}I-GDNF from GFRα-1, GFRα-2, and c-Ret binding sites. ^{125}I-GDNF binding to COS cells transiently transfected with the indicated plasmid DNA constructs was allowed to reach equilibrium (≈3 h). Cells were washed for the indicated periods of time or not washed (−) prior to cross-linking with EDAC. Phosphorimaging quantification of the bands indicated that 30 min after washing, 50% of ^{125}I-GDNF still remained bound to each of the three GDNF receptors.

In another experiment, we compared dissociation rates after binding of ^{125}I-GDNF to cells expressing c-Ret together with either GFRα-1 or GFRα-2 (Fig. 2B). ^{125}I-GDNF was allowed to bind until equilibrium, after which the binding mixture was removed and the cells were left in washing solution for different periods of time prior to cross-linking. Fifty percent of the ^{125}I-GDNF bound at the beginning of the experiment still remained associated with GFRα-1, GFRα-2, or c-Ret 30 min after washing (Fig. 2B), indicating relatively slow dissociation rates of these complexes. The binding affinity of GDNF to GFRα-1 and GFRα-2 was assessed in

a dose–response equilibrium binding assay by chemical crosslinking of radiolabeled GDNF to receptors expressed in COS cells (Fig. 3). From these data, a dissociation constant (K_d) of 4.7×10^{-10} M for GDNF binding to GFRα-1 and 2.1×10^{-9} M for GDNF binding to GFRα-2 could be obtained. The ability of GFRα-2 to mediate binding of GDNF to c-Ret was sensitive to treatment with phosphatidyl inositol-phospholipase C (PIPLC) (data not shown), indicating that GFRα-2, like GFRα-1, is also a GPI-anchored receptor.

Although a direct interaction between ^{125}I-GDNF and GFRα-3 could not be detected by chemical crosslinking (Fig. 4), coexpression of GFRα-3 and c-Ret in COS cells allowed affinity labeling of both receptors by ^{125}I-GDNF (Fig. 4). This result indicated that GDNF can bind to a GFRα-3/c-Ret complex but not the individual receptors.

Ligand-Stimulated c-Ret Tyrosine Phosphorylation Mediated by GFRα Receptors

Next, we examined whether the interaction of GDNF with c-Ret mediated by GFRα-2 and GFRα-3 enabled stimulation of tyrosine phosphorylation of the c-Ret receptor in intact cells. COS cells were transfected with a c-Ret expression construct in the presence or absence of expression plasmids for GFRα-1, GFRα-2, or GFRα-3. Forty eight hours after transfection, cells were either left unstimulated or stimulated with increasing amounts of GDNF, and cell lysates were analyzed for c-Ret tyrosine phosphorylation by immunoprecipitation with anti-c-Ret antibodies and Western blotting with anti-phosphotyrosine antibodies. Introduction of GFRα-1 and GFRα-2, but not GFRα-3, together with c-Ret in COS cells allowed the stimulation of c-Ret tyrosine phosphorylation by GDNF in a dose-dependent manner (Fig. 5A). GDNF was less efficient at stimulating c-Ret phosphorylation in cells expressing GFRα-2 compared to cells expressing GFRα-1 (Fig. 5A). With GFRα-3, no stimulation of c-Ret tyrosine phosphorylation could be seen with concentrations of GDNF up to 100 ng/ml. Reprobing of the blots with anti-c-Ret antibodies confirmed that the differences seen in phosphotyrosine levels were not due to different amounts of c-Ret protein in the lanes (Fig. 5A).

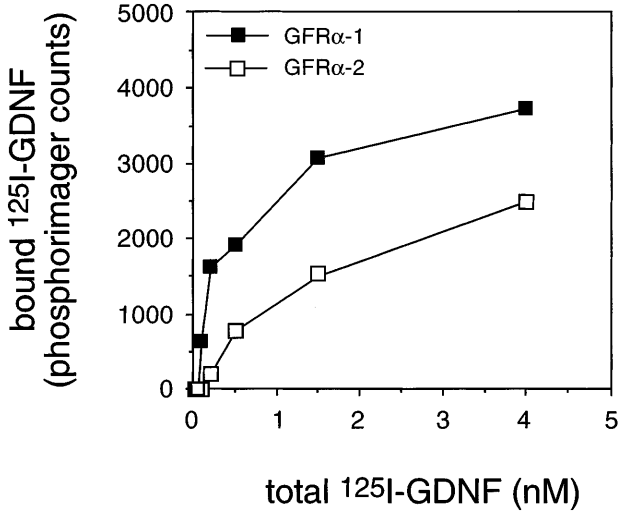


FIG. 3. Equilibrium binding of ^{125}I-GDNF to GFRα-1 and GFRα-2 expressed in COS cells. Increasing amounts of ^{125}I-GDNF were cross-linked to cell monolayers expressing GFRα receptors; bound ^{125}I-GDNF was quantified by phosphorimaging analysis of SDS/PAGE gels.

GFRα Receptors Negatively Modulate the Constitutive Levels of Tyrosine Phosphorylation of c-Ret Overexpressed in COS Cells

c-Ret showed high constitutive levels of tyrosine phosphorylation when overexpressed in COS cells even in the absence of ligand (Fig. 5A). Introduction of GFRα-1, GFRα-2, or GFRα-3 into COS cells overexpressing c-Ret reduced the constitutive level of tyrosine phosphorylation of this receptor without affecting its expression levels (Figs. 5 and 6). Tyrosine phosphorylation of c-Ret in unstimulated COS cells was likely due to receptor autophosphorylation since it was not seen in a c-Ret mutant (R897Q) with deficient tyrosine kinase activity (Fig. 5B). The inhibitory effect of GFRα receptors was dose-dependent and was detected at DNA ratios of GFRα to c-Ret in excess of 4 to 1 (Fig. 6). GFRα-1 and GFRα-2 were more effective than GFRα-3 at modulating c-Ret autophosphorylation (Fig. 6B). Interestingly, the level of c-Ret autophosphorylation was not affected after coexpression with a soluble GFRα-1 receptor construct lacking the GPI anchoring sequence (GFRα-1 GPI minus, Fig. 6), suggesting that GFRα receptors need to be anchored at the membrane to be able to affect constitutive c-Ret autophosphorylation in COS cells.

GFRα-1 and GFRα-2 Show Distinct Patterns of Expression in the Early Postnatal and Adult Brain

In a recent study, we reported complementary and overlapping domains of expression of GFRα-1, c-Ret, and GDNF, which revealed target-derived as well as paracrine trophic circuits in the mammalian central nervous system (Trupp *et al.*, 1997). Examination of sagittal sections through the adult rat brain hybridized with c-Ret and GFRα-2 riboprobes revealed strikingly segregated patterns of expression (Figs. 7A and 7B). Very low or no GFRα-2 expression was seen in several regions expressing high levels of c-Ret mRNA, including the substantia nigra, cerebellum, and cranial motor

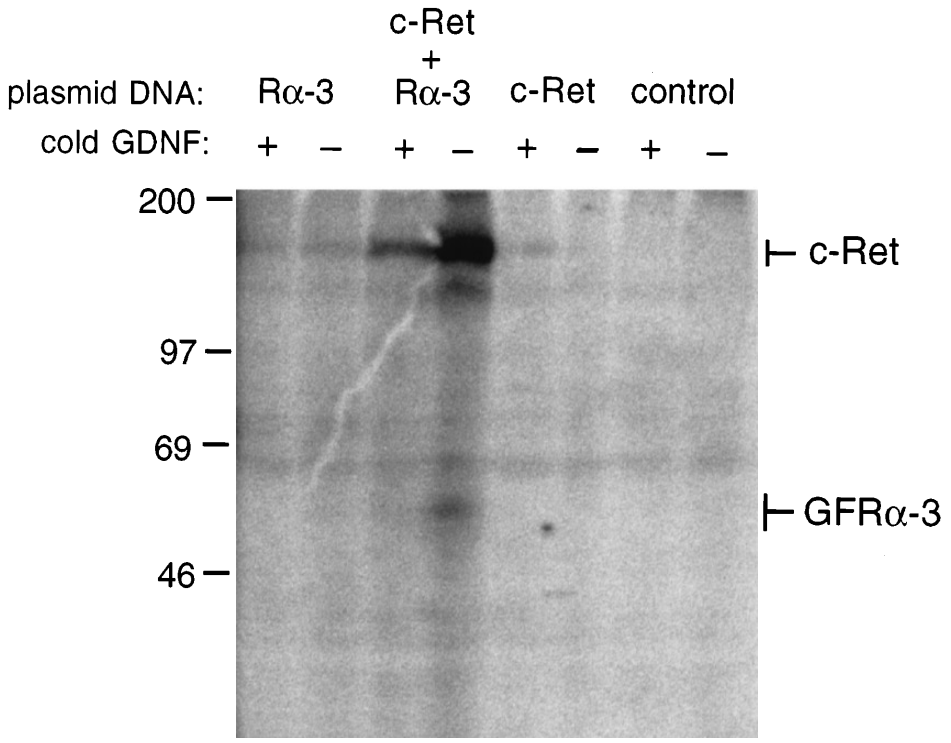


FIG. 4. GDNF can bind to a GFRα-3–c-Ret complex but not the individual receptors. ^{125}I-GDNF was allowed to bind to COS cells transiently transfected with the indicated plasmid DNA constructs in the presence (+) or absence (−) of an excess of unlabeled (cold) GDNF as indicated. Complexes between ^{125}I-GDNF and c-Ret or GFRα-3 are indicated.

nuclei which have been shown to express high levels of GFRα-1 (Figs. 7A and 7B). Expression of GFRα-2 and c-Ret mRNAs colocalized, however, in several other regions, including the glomerular layer of the olfactory bulb (Figs. 7A and 7B), which was seen to express no or very low levels of GFRα-1 (Trupp *et al.*, 1997). In the olfactory bulb, GFRα-2 showed a laminated pattern of expression restricted to distinct cellular layers, including the glomerular, mitral, and granule cell layers (Figs. 7C and 7D). Interestingly, partially overlapping and complementary expression patterns have been detected in the olfactory bulb for GDNF, GFRα-1, and c-Ret. GFRα-2 appeared to be colocalized with c-Ret in the glomerular layer, and with GDNF in the granule cell layer. GFRα-1 expression was only seen in the external and internal plexiform cell layers flanking the mitral cell layer, in which only GFRα-2 was expressed (Figs. 7C and 7D).

Other regions in the adult rat brain with high GFRα-2 expression included the inferior and superior colliculi, pineal gland, lateral septum, and cortex (Fig. 7B). Expression of GFRα-2 in many of these regions could also be detected in the early postnatal brain (Figs. 8A–8C). In addition to these, GFRα-2 expression was also seen in various other structures of the postnatal day 7 (P7) rat brain, including the striatum and olfactory tubercule (Fig. 8A), the hippocampus (Fig. 8B), as well as several nuclei in the ventral midbrain, including the ventral tegmental area and lateral fields of the substantia nigra (Fig. 8C). Thus, although adult substantia nigra pars compacta neurons appear not to express GFRα-2, this receptor is transiently expressed at low to moderate levels early in postnatal development in several regions of the ventral midbrain, most notably in the lateral fields of the substantia nigra, which contain dopaminergic neurons that also express c-Ret.

GDNF, c-Ret, and GFRα-1 show regulated patterns of expression in the adult rat brain after kainic acid stimulation (Trupp *et al.*, 1997). Like GFRα-1, expression of GFRα-2 mRNA was increased in the dentate gyrus after kainic acid stimulation (Fig. 9). Unlike GFRα-1, however, the regulation of GFRα-2 mRNA by kainic acid was delayed, peaking at 12 h instead of 4 h, and more transient, returning to control levels by 24 h as opposed to the sustained expression of GFRα-1 mRNA. The greatest increase in GFRα-2 expression 12 h after kainic acid treatment was seen in several thalamic nuclei (Fig. 9), regions that do not express elevated levels of GFRα-1 mRNA after this treatment (Trupp *et al.*, 1997). The induced GFRα-2 mRNA expression in these nuclei was also transient and decreased back to near control levels 24 h after the treatment.

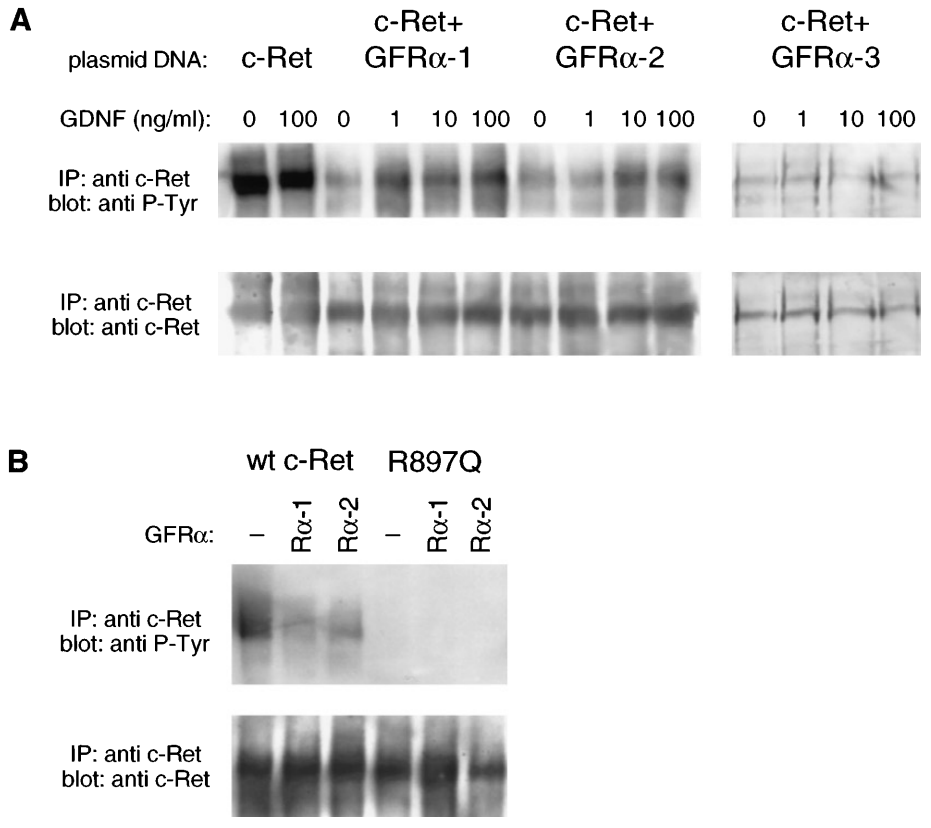


FIG. 5. GFRα receptors allow ligand stimulation of c-Ret tyrosine phosphorylation. (A) In the absence of GPI-linked receptors, c-Ret showed a high level of constitutive tyrosine phosphorylation which was unaffected by ligand treatment. Introduction of GFRα-1 and GFRα-2, but not GFRα-3, receptors allowed dose-dependent ligand stimulation of c-Ret tyrosine phosphorylation. The lower panels show the reprobing of the corresponding filters with an anti-c-Ret antibody. In the experiment shown, plasmid DNA of GFRα receptors and c-Ret were transfected at a 10:1 ratio. (B) Constitutive phosphorylation in COS cells is due to receptor autophosphorylation. Wild-type c-Ret (wt c-Ret) but not a kinase-deficient c-Ret mutant (R897Q) showed high levels of constitutive phosphorylation after transient expression in COS cells. The lower panel shows the reprobing of the filter with an anti-c-Ret antibody.

Widespread Peripheral Expression of GFRα-3

Unlike GFRα-1 and GFRα-2, GFRα-3 mRNA was not detected in hippocampus before or after kainate treatment (data not shown). Also in contrast to GFRα-1 and GFRα-2, GFRα-3 mRNA expression could not be detected by RNAse protection assay in total homogenates of postnatal or adult rat brain (Fig. 10A). Low levels of GFRα-3 expression were detected in homogenates of embryonic rat brain (Fig. 10A). By *in situ* hybridization, GFRα-3 mRNA was, however, detected in very restricted nuclei of the adult rat brain, including the thalamic parafascicular nucleus, the medial preoptic nucleus of the hypothalamus and the amygdalohippocampal anterolateral nucleus (data not shown). High levels of GFRα-3 mRNA expression were found in P1 mouse dorsal root ganglia (DRG) and lower levels in P1 mouse spinal cord (Fig. 10A). GFRα-3 showed a widespread pattern of expression in nonneuronal tissues, including embryonic, but not adult, pancreas and skeletal muscle (data not shown and Fig. 10B), adult spleen, ovary, and heart (data not shown), and late postnatal salivary gland, liver, lung, and kidney (Fig. 10B). In kidney, GFRα-3 and GFRα-1 showed opposing patterns of developmental regulation (Fig. 10B).

DISCUSSION

Structural Similarities and Differences between GFRα Receptors

In this study, we investigated the interactions of c-Ret and GDNF with members of the GFRα receptor family, including a novel member termed GFRα-3. GFRα-2 and GFRα-3 have structural and functional features in common with GFRα-1. Regions of similarity between the amino acid sequences of these three receptors clustered

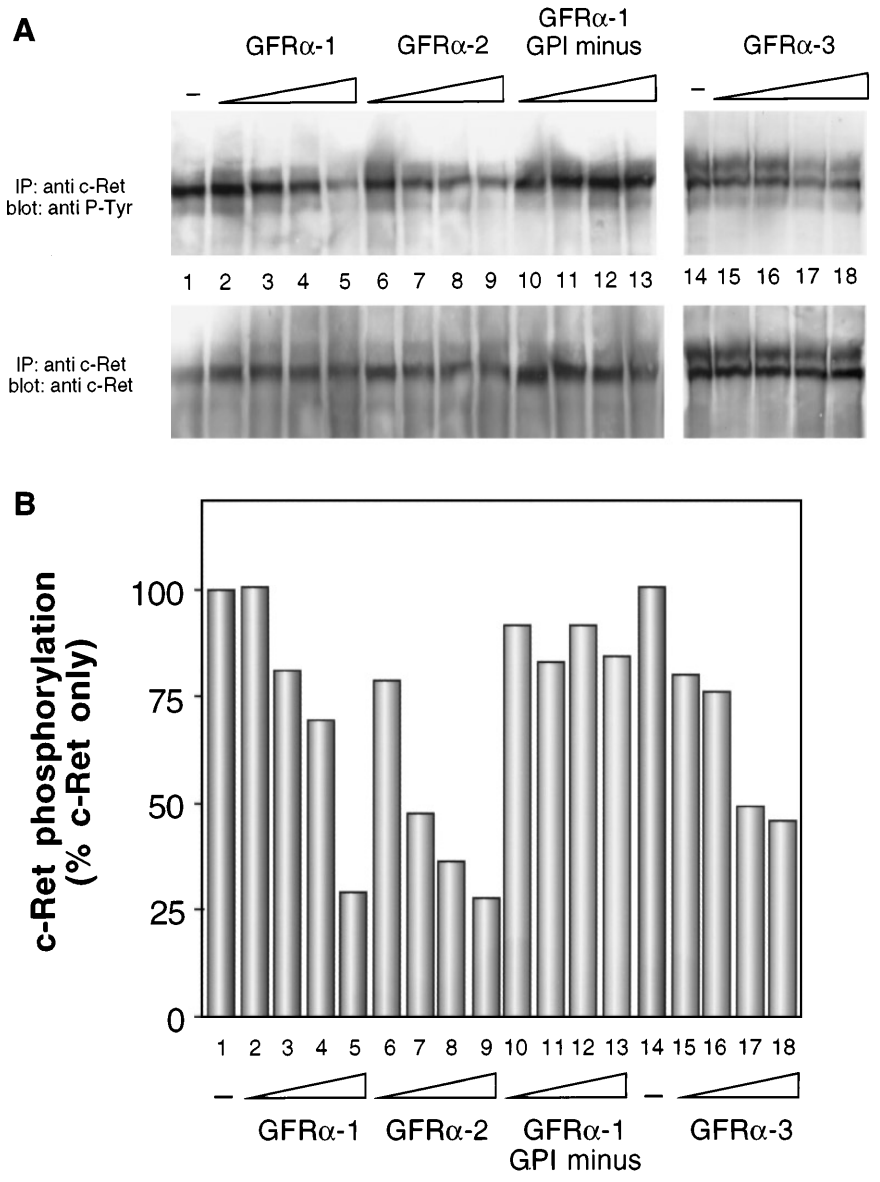


FIG. 6. Negative modulation of c-Ret autophosphorylation by GFRα receptors in COS cells. (A) Dose–response analysis of GFRα-1, GFRα-2, and GFRα-3 inhibition of ligand independent c-Ret autophosphorylation. COS cells were transfected with 1 μg of c-Ret plasmid DNA in the absence (−) or presence of the indicated GFRα plasmid DNAs at increasing ratios with respect to c-Ret; i.e., 1:1 (lanes 2, 6, 10, and 15), 4:1 (lanes 3, 7, 11, and 16), 10:1 (lanes 4, 8, 12, and 17), and 20:1 (lanes 5, 9, 13, and 18). The final amount of plasmid DNA was adjusted in each case to 20 μg with empty pCDNA3 vector DNA. Note that a GFRα-1 construct lacking the GPI-anchor domain (GPI minus) was unable to modulate constitutive c-Ret autophosphorylation in COS cells (lanes 10 to 13). The lower panel shows the reprobing of the corresponding filters with an anti-c-Ret antibody. (B) Quantitative analysis of c-Ret tyrosine phosphorylation levels in COS cells. Blots were scanned in a Storm 840 fluorimager (Molecular Dynamics), quantitative analysis was made with ImageQuant software. The same analysis applied to the reprobed filter showed no significant differences in the levels of c-Ret between the different lanes.

in discrete segments, particularly in the central portion of their primary sequences. These may represent domains important for the correct folding and three-dimensional conformation of members of this receptor family. Although both GFRα-1 and GFRα-2 were able to bind GDNF, this ligand appeared to interact less efficiently with GFRα-2, as indicated by cross-linking and phosphorylation experiments. Dose–response binding assays indicated an affinity approximately fivefold lower for GDNF binding to GFRα-2 compared to GFRα-1. Variable regions between GFRα-1 and GFRα-2 may contribute to the formation of similar but distinct bind-

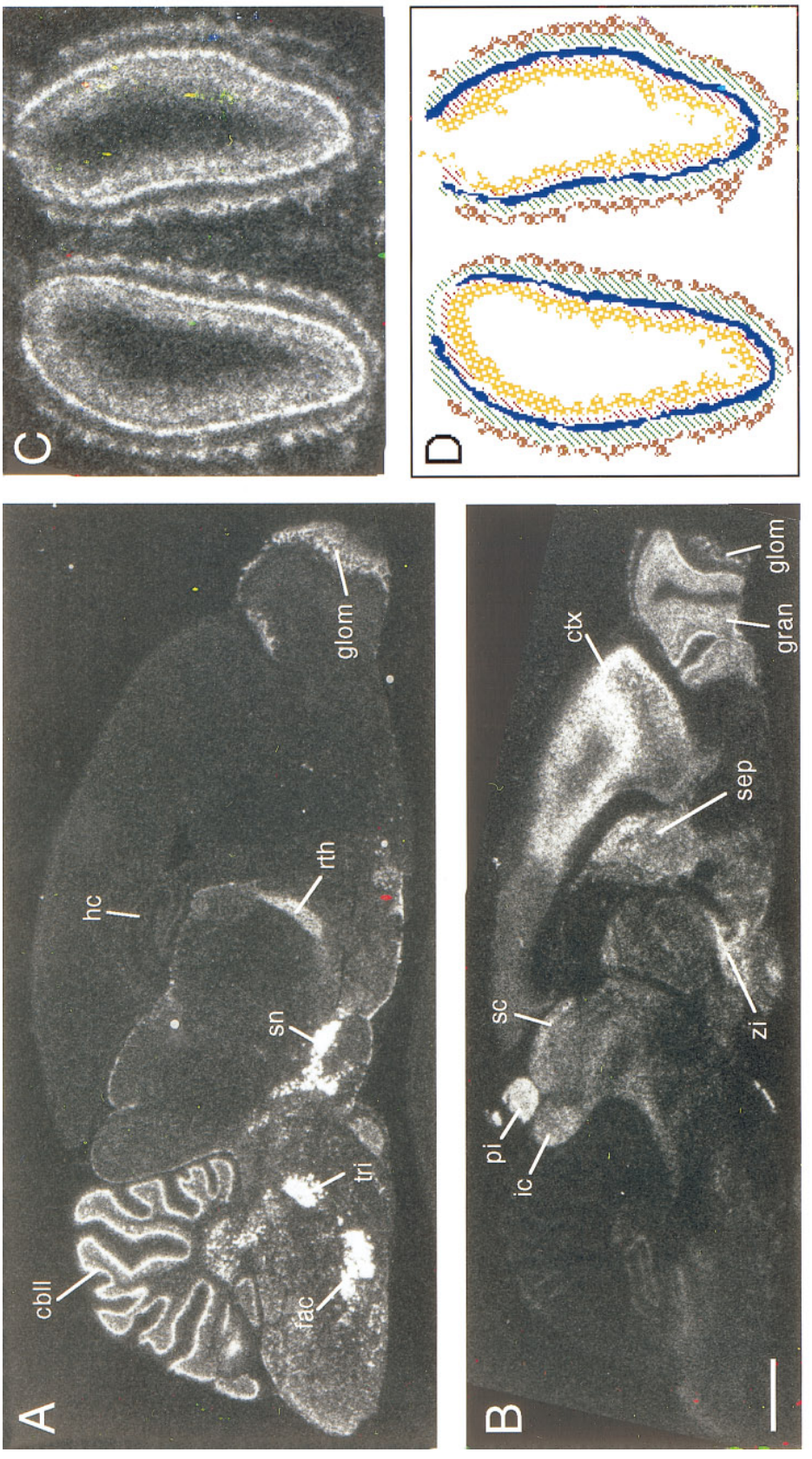
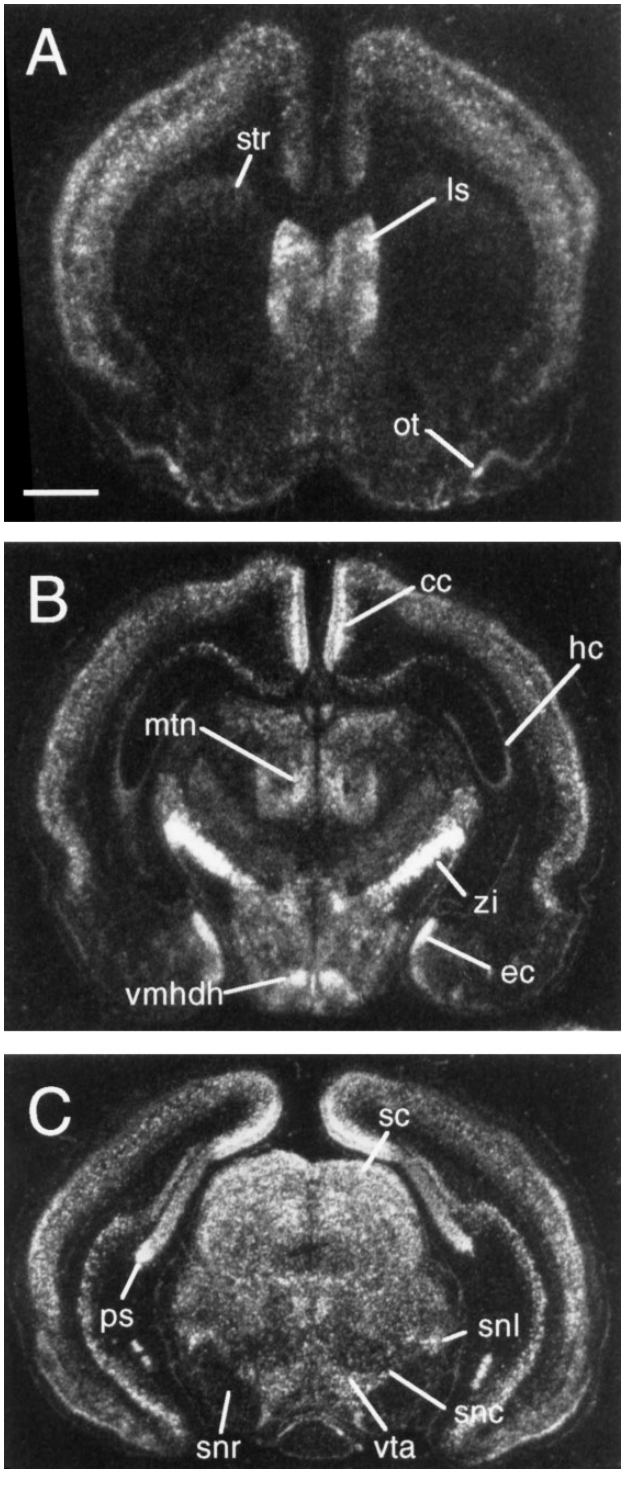


FIG. 7. Expression of c-Ret and GFRα-2 mRNA in the adult rat brain. (A) Sagittal section through the adult rat brain hybridized with a riboprobe complementary to c-*Ret* mRNA. Strong labeling for c-*Ret* mRNA is seen in the cerebellum (cbll), substantia nigra (sn), and facial (fac) and trigeminal (tri) motor nuclei. c-*Ret* mRNA was also found in the reticular thalamic nucleus (rth) and in the glomerular layer of the olfactory bulb (glom). Very low levels of c-*Ret* mRNA were expressed in the hippocampus (hc). (B) Sagittal section through the adult rat brain hybridized with a riboprobe complementary to GFRα-2 mRNA. Strong expression was seen in the pineal gland (pi), cortex (ctx), zona incerta (zi), inferior (ic) and superior (sc) colliculi, septum (se), and in several layers of the olfactory bulb, including the glomerular (glom) and granule cell (gran) layers. (C) Coronal section through the olfactory bulb showing expression of GFRα-2 mRNA in the glomerular, mitral, and granule cell layers. (D) Schematic diagram of the olfactory bulb as shown in C, indicating the glomerular (brown), external plexiform (green), mitral (blue), internal plexiform (red), and granule (yellow) cell layers. GFRα-2 colocalized with c-Ret in the glomerular layer and with GDNF in the granule cell layer. The mitral cell layer expressed only GFRα-2, while expression of GFRα-1 was found predominantly in the internal and external plexiform layers (Trupp *et al.*, 1997). Scale bar, 1.56 mm in A; 1.64 mm in B; 1.63 mm in C.

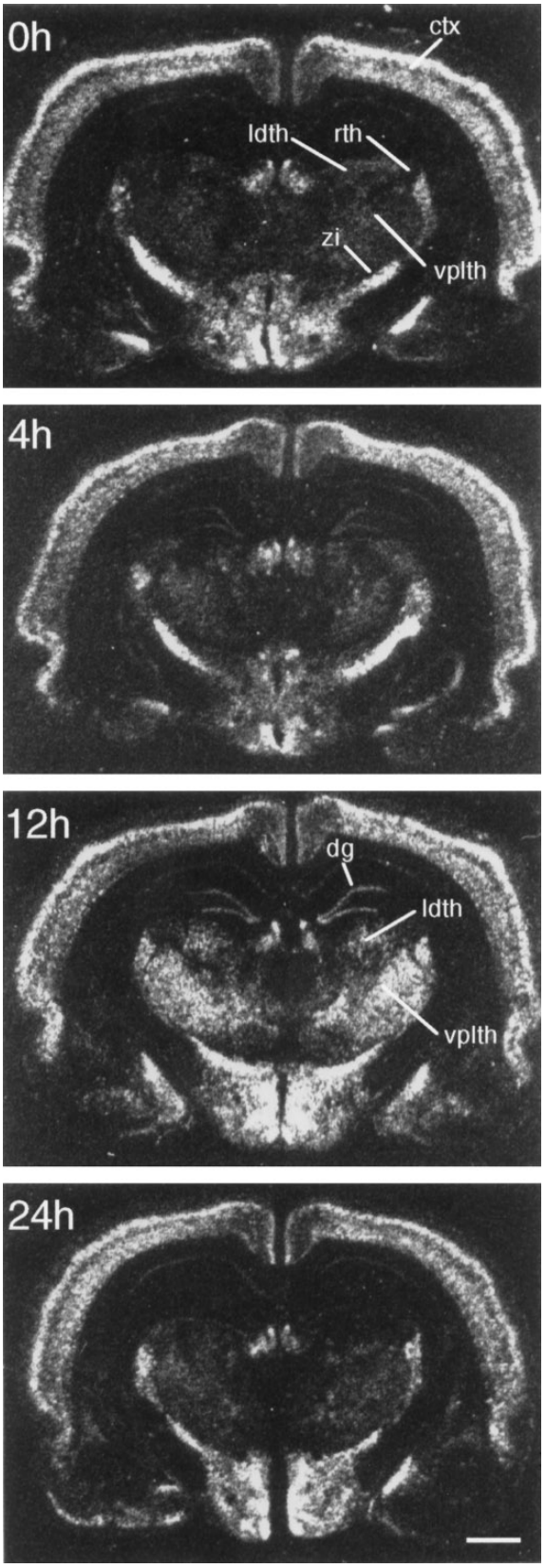
ing epitopes to GDNF and could therefore account for the differences observed between the two receptors in their interaction with this ligand. Unlike GFRα-1 and GFRα-2, GFRα-3 was unable to bind GDNF directly but required coexpression with c-Ret. GFRα-3 is a more divergent member of the GFRα family, particularly in the N- and C-terminal regions of the molecule. In the latter, GFRα-3 lacks two cysteine residues as well as the intervening domains which are present in both GFRα-1 and GRFα-2. This region could therefore be of importance for the direct association of GFRα receptors with GDNF. Despite its ability to mediate crosslinking of GDNF to c-Ret, GFRα-3 was unable to mediate c-Ret activation by GDNF in COS cells in the range of ligand concentrations tested. These data indicate that, although some c-Ret molecules can be recruited to the ligand–receptor complex by GFRα-3, this interaction is not strong enough to stabilize a sufficient number of c-Ret dimers, a prerequisite for receptor activation and downstream signaling. Although higher concentrations of GDNF may result in the activation of c-Ret in the presence of GFRα-3, together, our observations suggest the existence of alternative cognate ligands for the GFRα-3 receptor distinct from GDNF.



Mechanisms of Receptor Activation

Using soluble extracellular domains, two recent studies were unable to detect an interaction between GFRα-2 and GDNF. However, Sanicola *et al.* (1997) did observe GDNF binding to soluble GFRα-2 in the presence of soluble c-Ret extracellular domain. Using chemical crosslinking, here we could show a specific and direct interaction between GDNF and GFRα-2 in intact cells in the absence of c-Ret, suggesting that localization of GFRα-2 at the cell membrane may be important for ligand binding. GFRα-2 was able to mediate c-Ret activation by GDNF (this study and Baloh *et al.*, 1997; Sanicola *et al.*, 1997; Suvanto *et al.*, 1997), indicating that the interaction between GDNF and GFRα-2 may be physiologically relevant. This is particularly interesting given the widespread pattern of expression of GFRα-2,

FIG. 8. Expression of GFRα-2 mRNA in P7 rat brain. (A) Coronal section through the septal level of the brain showing GFRα-2 mRNA labeling in the lateral septum (ls) and in the olfactory tubercule (ot). Weak labeling was also seen in the striatum (str). (B) A more posterior coronal section showing strong GFRα-2 mRNA labeling in the cingulate cortex (cc), zona incerta (zi), endopiriform cortex (ec), and the dorsomedial part of the ventromedial hypothalamic nucleus (vmhdh). Weaker but detectable labeling was also seen in medial thalamic nuclei (mtn) and in the hippocampus (hc) which included scattered extra pyramidal cells labeled for GFRα-2 mRNA. (C) A coronal section of a P7 rat brain at the level of the midbrain showing strong GFRα-2 expression in the superior colliculus (sc) and the presubiculum (ps). Moderate labeling was also seen in lateral fields of the substantia nigra (snl) and ventral tegmental area (vta). Scale bar, 2.1 mm in A, B; 760 nm in C.



and indicates a broader range of activities for GDNF than previously anticipated, particularly under pharmacological situations.

Redundancy and promiscuity in ligand–receptor interactions appear to be the rule rather than the exception in many families of growth factors and receptors. To which extent these interactions represent physiologically important events is a crucial but difficult question, which will require examination of the responses of different primary cell types expressing individual receptors and ultimately gene targeting studies for its clarification. Our observations suggest a graded response of GFRα receptors to GDNF such as GFRα-1>GFRα-2>GFRα-3. GFRα-1 may thus mediate responses to low GDNF concentrations, whereas GFRα-2 and GFRα-3 may require elevated levels of ligand and/or c-Ret coexpression in order to mediate a response. This may constitute a mechanism for triggering differential responses to GDNF gradients which may have important roles in morphogenesis and differentiation.

We and others have previously reported data which demonstrated that c-Ret is a functional receptor for GDNF (Durbec *et al.*, 1996; Trupp *et al.*, 1996; Vega *et al.*, 1996; Worby *et al.*, 1996). Our new results are in agreement with other reports which indicated the necessity of GPI-linked subunits as coreceptors for GDNF binding to and activation of c-Ret (Jing *et al.*, 1996; Treanor *et al.*, 1996). All members of the GFRα receptor family are expressed in MN1 cells (M.T. and C.F.I., unpublished), a motorneuron cell line that expresses c-Ret and displays biochemical and biological responses to GDNF (Trupp *et al.*, 1996). Moreover, expression of GFRα-1 was also found in a line of NIH3T3 fibroblasts stably transfected with a c-Ret expression construct (M.T. and C.F.I., unpublished) used in previous studies to demonstrate the necessity of the c-Ret receptor in GDNF signalling (Trupp *et al.*, 1996). Thus, all the evidence available so far indicates that GPI-anchored receptor components are required for activation of the c-Ret receptor tyrosine kinase by GDNF. However, it is still unclear whether GDNF first binds to GPI-linked receptors and subsequently this complex interacts with c-Ret as proposed by some models (Jing *et al.*, 1996; Treanor *et al.*, 1996), or whether GDNF stabilizes preformed complexes be-

FIG. 9. Kainic acid induced neuronal activation upregulates GFRα-2 mRNA levels in the adult rat brain. Pronounced elevation of GFRα-2 mRNA expression was seen in the ventroposteriomedial (vpmth), ventroposterolateral (vplth), and latrodorsal (ldth) thalamic nuclei 12 h after kainic acid stimulation. Increased levels of GFRα-2 mRNA were also seen in the dentate gyrus (dg), peaking at 12 h and subsiding by 24 h. Scale bar, 1.6 mm.

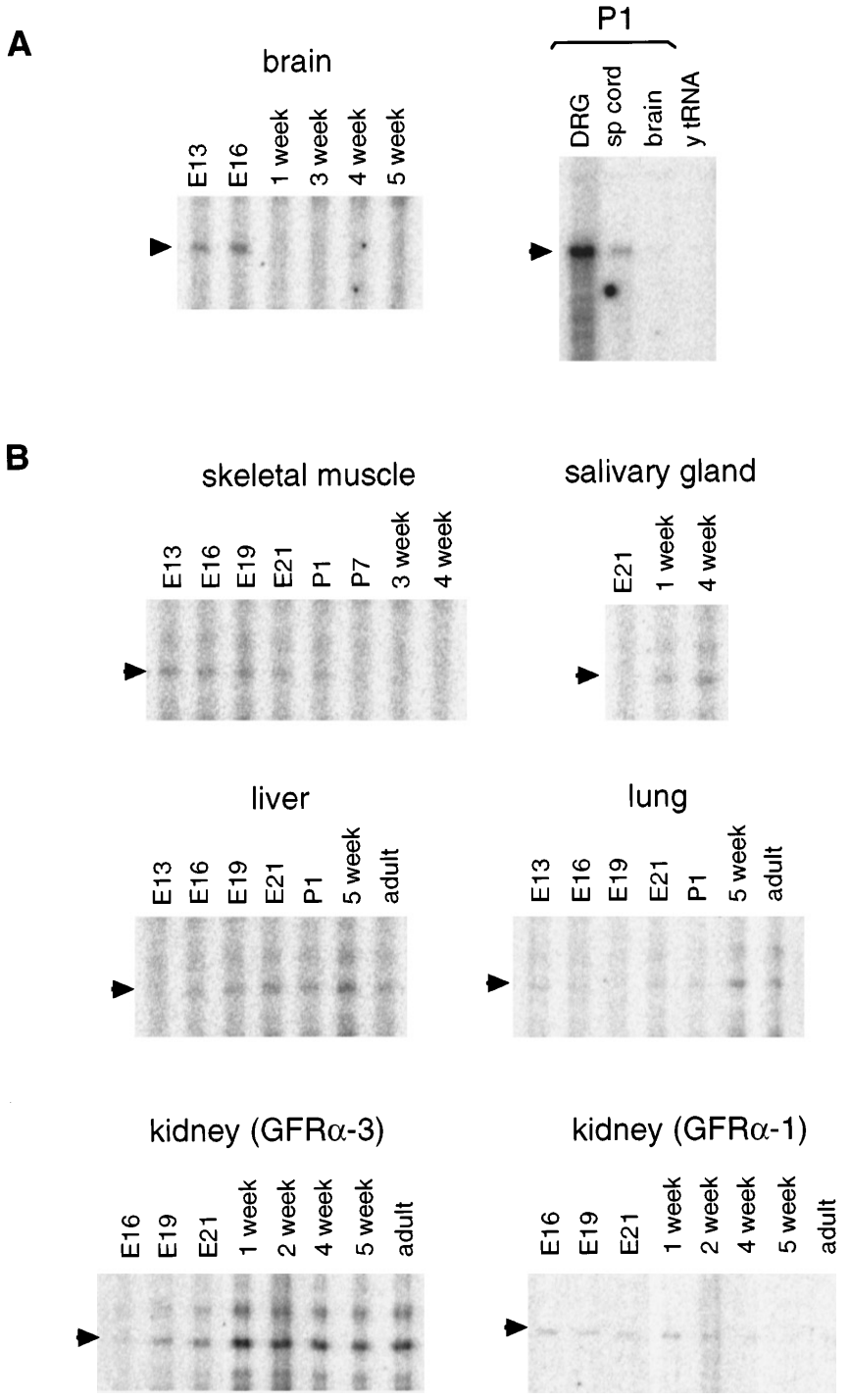


FIG. 10. GFRα-3 mRNA expression analyzed by RNAse protection assay. (A) Low and transient expression of GFRα-3 in embryonic rat brain (left panel). High expression in early postnatal mouse DRG and lower expression in mouse spinal cord (right panel). yeast transfer RNA (y tRNA) was used as negative control. (B) Widespread expression of GFRα-3 mRNA in rat peripheral organs. All panels correspond to GFRα-3, except where indicated. Equal RNA loading was confirmed in each case by including a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe with every sample (not shown).

tween c-Ret and GPI-anchored GDNF receptors at the cell membrane. In fact, both GFRα-1 and GFRα-2 have been detected in c-Ret immunoprecipitates from unstimulated cells coexpressing both receptors (Klein *et al.*, 1997; Treanor *et al.*, 1996) and soluble c-Ret extracellular domain is able to interact with soluble GFRα-1 *in vitro* (Sanicola *et al.*, 1997). These data suggest that preformed complexes between c-Ret and GPI-linked receptors are present at the cell membrane in the absence of ligand. The fact that GDNF could be crosslinked to a GFRα-3/c-Ret complex but not to the individual receptors also supports a GFRα–c-Ret interaction. However, while it has been suggested that GFRα-1 and possibly GFRα-2 may function as ligand presenting receptors in a trans-acting fashion (Trupp *et al.*, 1996), this appears not to be possible for GFRα-3.

Our data showing inhibition of constitutive c-Ret tyrosine phosphorylation in unstimulated COS cells by GFRα receptors also suggest a ligand-independent interaction between GPI-linked receptors and c-Ret, an observation that might be of importance for the regulation of the activity of oncogenic forms of c-Ret. Interestingly, this effect required GPI-anchorage of GFRα-1 to the cell membrane, perhaps because restricting the mobility of GFRα receptors to the two-dimensional plane of the membrane facilitates their interaction with c-Ret. We have also shown that constitutive phosphorylation of c-Ret in unstimulated COS cells was due to receptor autophosphorylation, suggesting the spontaneous formation of c-Ret homodimers in transfected COS cells. Although the effect of GFRαs on c-Ret autophosphorylation have so far only been observed in COS cells, it nevertheless suggests that c-Ret and GFRα receptors can interact in the absence of ligand. As a consequence of that interaction, constitutive activation of the c-Ret tyrosine kinase is inhibited, perhaps because the association of c-Ret with GFRα receptors interferes with the spontaneous formation of c-Ret homodimers. We speculate that, in addition to allowing ligand-dependent activation of c-Ret, GFRα receptors could also provide a gain control mechanism to increase the signal-to-noise ratio of the response to ligand.

With the cloning of NTN, a protein with striking structural and functional similarities to GDNF (Kotzbauer *et al.*, 1996), a family of GDNF-related molecules is beginning to emerge. Recent data demonstrating an interaction between NTN and GFRα-2 (Baloh *et al.*, 1997; Buj-Bello *et al.*, 1997; Klein *et al.*, 1997) indicate that GFRαs serve as coreceptors for several members of the GDNF ligand family. It will therefore be of interest to investigate whether NTN, or the third member of the GDNF ligand family, persephin (PSP) (Milbrandt *et al.*, 1998), are ligands of GFRα-3. The fact that all three GFRα receptors were able to mediate GDNF binding to c-Ret suggests that GPI-anchored receptors will not be the sole determinants of the specificity of the actions of the different members of the GDNF family. Moreover, it is not yet clear whether all GDNF family members will only signal through c-Ret or whether other c-Ret-related or unrelated signalling receptors may exist. Some of the evidence presented here encourages the notion of additional signalling components in the receptor system for this family of ligands. The fact that both GFRα-1 and GFRα-2 appear to be much more widely expressed in the brain than c-Ret, for example, suggests that these may associate with additional signal-transducing molecules. Alternatively, as we proposed recently, GPI-anchored receptors may function in a soluble form or to concentrate and present ligands to afferent c-Ret-expressing cells (Trupp *et al.*, 1997). In agreement with the latter possibilities, we now find coexpression of GFRα-2 and GDNF in several regions, such as the cingulate cortex and the granule cell layer of the olfactory bulb. Interestingly, high levels of NTN mRNA expression appear to colocalize with GFRα-2, but not with GDNF, GFRα-1, or c-Ret, in the pineal gland and with GFRα-3 in the salivary gland (S. Eketjäll, M.T., and C.F.I., unpublished), indicating that coexpression of GDNF family ligands and GFRα receptors is a common feature of the adult nervous system.

Distinct Patterns of Expression

A dynamic pattern of GFRα-2 expression was seen during the postnatal development of the rat brain, with several structures showing transient GFRα-2 expression during early postnatal development, including hippocampus, striatum, and some ventral midbrain nuclei. Interestingly, although expressed below detection levels in the adult hippocampus, GFRα-2 mRNA levels could be induced by kainic acid treatment, which also augments expression of GFRα-1 (Trupp *et al.*, 1997), suggesting an activity regulated trophic response that can have a role in neuronal plasticity. Unlike GFRα-1, however, a pronounced increase in GFRα-2 mRNA expression was seen in thalamic nuclei after kainic acid treatment, indicating differences in the dynamic expression of these two receptors after neuronal activation. The absence of GFRα-2 expression in the adult substantia nigra and cranial motor nuclei described here and in a recent publication (Widenfalk *et al.*, 1997) is intriguing and suggests that the well documented activities of GDNF on cells from those structures are primarily mediated by GFRα-1. GFRα-2 was also barely detectable in the cerebellum, where high levels of both c-Ret and GFRα-1 are expressed (Trupp *et al.*, 1997). However, GFRα-2 was

expressed in several regions previously shown to express c-Ret but not GFRα-1 (Trupp *et al.*, 1997), such as hippocampal extrapyramidal cells and the glomerular cell layer of the olfactory bulb. In the olfactory bulb, highly specific patterns of expression are emerging for c-Ret and its ligands and coreceptors. In particular, GFRα-2 was found expressed either alone, as in the mitral cell layer, or together with GDNF, as in the granule cell layer, or with c-Ret, as in the glomerular cell layer, suggesting that these molecules mediate paracrine and reciprocal trophic actions between the different neuronal populations of the olfactory bulb. In other parts of the brain, such as the lateral septum, and the superior and inferior colliculi, GFRα-1 and GFRα-2 expression overlap.

In contrast to GFRα-1 and GFRα-2, expression of GFRα-3 was predominantly absent in the adult brain, even after acute neuronal activation by kainic acid. However, high levels of GFRα-3 expression were seen in early postnatal DRG, suggesting that this receptor may play a role in the survival or differentiation of peripheral neurons. Widespread expression of GFRα-3 was observed in many peripheral tissues. Although in some cases, such as developing skeletal muscle which also produces GDNF, shed GFRα receptors may have a neurotrophic function by facilitating the capture of ligand by incoming nerve terminals, in other organs GFRα-3 may play nonneuronal roles in the morphogenesis or maintenance of a differentiated state.

In conclusion, our data demonstrates the existence of an extended family of receptors that control ligand-dependent and independent activation of the c-Ret tyrosine kinase. Further studies will be required to address the physiological importance of these interactions as well as the role played by GDNF and the different GFRα receptors in the various human diseases caused by alterations in the c-*Ret* gene.

EXPERIMENTAL METHODS

DNA Cloning, Sequence Analysis, and Site-Directed Mutagenesis

A full-length GFRα-1 cDNA was isolated by PCR from rat brain cDNA using primers 5′-CGGCGGCACCATGTTCCTAG-3′ and 5′-TCCCGGATGCAGCTACGAC G-3′. For isolation of a GFRα-2 cDNA, PCR was performed on human fetal brain cDNA (Clontech) using primers 5′-ATGGATCCGCAACCTGAATGACAACTGC-3′ and 5′CCGAATTCAGTTGGGCTT CTCCTTGTC-3′ based on Genbank EST H12981. A fragment of 250 bp was obtained which was subsequently used to screen a rat postnatal day 7 (P7) brain cDNA library. A 3-kb cDNA insert was isolated from a purified hybridizing phage. A full-length mouse GFRα-3 cDNA was obtained by extending the mouse EST AA049894 with a RACE product obtained by priming mouse E15 cDNA (Clontech) with primer 5′-AGCTGGGCTAGGCAGAGGTGGCG CTGGC-3′ and a kit of reagents from Clontech. Both strands of these cDNA clones were sequenced in an ABI 310 automatic sequencer (Perkin–Elmer). Interestingly, efficient heterologous expression of the GFRα-3 cDNA clone in transfected cells required removal of 5′ and 3′ untranslated flanking sequences and modification of the nucleotides immediately upstream of the initiation codon to better conform with Kozak consensus sequences. Alignments, localization of N-glycosylation sites, and DNA restriction mapping was done using programs from the Genetics Computer Group (Genetics Computer Group, 1994). Full-length GFRα-1, GFRα-2, and GFRα-3 cDNAs were subcloned into pCDNA3 (Invitrogen) for transient expression in transfected cells. A full-length human c-Ret cDNA (long isoform) (Takahashi *et al.*, 1988) subcloned in pcDNA3 was obtained from Vassilis Pachnis (National Institute of Medical Research, London, UK). The mutant c-Ret (R987Q) was produced by oligonucleotide-mediated site-directed mutagenesis (Kunkel *et al.*, 1987). This mutation, found in patients with Hirschsprung's disease, replaces Gln for Arg at position 897 in the c-Ret tyrosine kinase domain and results in inactivation of the kinase.

In Vitro Translation and Cell Transfections

Protein expression from GFRα cDNA constructs was checked by coupled *in vitro* transcription/translation using a kit of reagents from Promega. COS cells grown to about 60% confluency were transfected with a constant total of 20 μg plasmid DNA per 100-mm dish using the DEAE dextran-chloroquine protocol. Transfected cells were assayed 48 to 72 h after transfection.

Cross-Linking and Phosphorylation Assays

All binding and biochemical studies were carried out with recombinant rat GDNF produced in Sf21 insect cells and purified as previously described (Trupp *et al.*, 1995). GDNF was iodinated as previously described (Trupp *et al.*, 1995) to a specific activity of 0.5–2 $\times$ 10^8 cpm/μg. ^{125}I-GDNF at 10 ng/ml was allowed to bind to cell monolayers at 4°C during 3 h and was subsequently cross-linked using ethyl-dimethylaminopropyl carbodiimide (EDAC) as cross-linking agent supplemented with Sulfo-NHS (Pierce) during 30 min at 4°C. Unlabeled GDNF was used at 50$\times$ molar excess. Receptor

complexes were fractionated by SDS/PAGE and visualized by autoradiography in a Storm 840 phosphorimager (Molecular Dynamics). For dissociation binding assays, ^{125}I-GDNF was allowed to bind to cell monolayers for 3 h, after which the binding mixture was removed and replaced with ice-cold binding buffer. The cells were then left in binding buffer at 4°C for different periods of time prior to cross-linking.

For PI-PLC treatment, cell monolayers were washed and then incubated with 0.3 U/ml PI-PLC (Boehringer Manheim) in cell culture medium for 30 min at 37°C, followed by affinity labeling as above. Saturation binding assays were performed essentially as previously described (Trupp *et al.,* 1996). Briefly, increasing concentrations of ^{125}I-GDNF were incubated with cell monolayers and, following 3 h incubation at 4°C, receptor–ligand complexes were crosslinked with EDAC for 30 min at 4°C, fractionated by SDS/PAGE, and visualized by autoradiography. Bands corresponding to GDNF receptor complexes were quantified in a Storm 840 phosphorimager using ImageQuant v1.1 software (Molecular Dynamics), and the corresponding values were plotted as a function of the total amount of ^{125}I-GDNF added.

Assay of steady-state levels of c-Ret tyrosine phosphorylation in intact cells was performed as previously described. Briefly, cell monolayers were exposed to different concentrations of GDNF for different periods of time. Nonidet P-40 (NP-40) cell lysates, prepared with protease and tyrosine phosphatase inhibitors, were immunoprecipitated with antibodies against the long isoform of c-Ret (Santa Cruz). Immunoprecipitates were fractionated by SDS/PAGE and analyzed by Western blotting with anti-phosphotyrosine antibodies (Upstate Biotechnology). Western blots were developed using alkaline phosphatase-conjugated secondary antibodies followed by chemifluorescence detection (Amersham), and analyzed in a Storm 840 fluorimager (Molecular Dynamics) using ImageQuant v1.1 software.

In Situ Hybridization and RNAse Protection Assay

c-Ret and GFRα-2 riboprobes were labeled with ^{35}S-UTP or ^{33}P-UTP using linearized template DNA fragments and reagents for *in vitro* transcription from Promega. For *in situ* hybridization, 14-μm sections were thawed onto 3-α-aminopropyl ethoxysilane-coated slides for hybridization with radiolabeled probes as follows. Following fixation in 4% paraformaldehyde for 15 min, slides were rinsed once in PBS and twice in distilled water. Tissue was deproteinated in 0.2 M HCl for 10 min, acetylated with 0.25% acetic anhydride in 0.1 M ethanolamine for 20 min, and dehydrated with increasing concentrations of ethanol. Slides were incubated 16 h in a humidified chamber at 58°C with 8×10^5 cpm of probe in 300 ml of hybridization cocktail (50% formamide, 20 mM Tris–HCl (pH 7.6), 1 mM EDTA, pH 8.0, 0.3 M NaCl, 0.1 M dithiothreitol, 0.5 μg/ml yeast tRNA, 0.1 μg/ml poly(A)-RNA, 1× Denhardt's solution and 10% dextran sulphate). Slides were first washed at room temperature in Formamide:SSC (1:1) followed by 30 min at 65°C in 1× SSC. Single-stranded RNA was digested by RNAse treatment (10 μg/ml) for 30 min at 37°C in 0.5 M NaCl, 20 mM Tris–HCl, pH 7.5, 2 mM EDTA. Tissue was washed twice with 1× SSC at 65°C for 30 min before dehydration in ethanol and air drying. Slides were either exposed to β-max X-ray film (Amersham, UK) for 10–20 days or dipped in NTB-2 photoemulsion diluted 1:1 in water (Eastman-Kodak) exposed at 4°C for 3–5 weeks, developed with D19 (Eastman-Kodak Co.), fixed with A1–4 (Agfa Gevaert), and counterstained with cresyl violet. RNAse protection assay (RPA) was performed using reagent kits from Promega (*in vitro* transcription) and Ambion (RPA). In all cases, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe was included with the sample. This signal was used to confirm equal RNA loading in each of the lanes of a given experiment.

Kainic Acid Stimulation

Adult male Wistar rats (150–170 g) received bilateral ICV injections of kainic acid (0.35 μg/0.5 ml; Sigma, St. Louis, MO) since in our experience this offers the advantage of a standardized latency of initiation and intensity of seizure activity with abrupt termination of status epilepticus, contributing notably to a reduction of interanimal variability. Animals were mounted in a stereotactic frame and injected using the following stereotaxic coordinates: AP 0.2, L. 1.5, V 4.2 from the bregma. At the indicated time points animals were sacrificed by decapitation after ether anesthesia, the brains removed, frozen in −40°C isopentane, stored at −70°C, and cryosectioned for *in situ* hybridization.

Note added in proof: The cloning of GFR-α3 has recently been reported by several other groups; Jing *et al.* (1997) *J. Biol. Chem.* **272:** 33111–33117; Masure *et al.* (1998) *Eur. J. Biochem.* **251:** 622–630; Naveilhan *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95:** 1295–1300; Worby *et al.* (1998) *J. Biol. Chem.* **273:** 3502–3508.

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