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CLONING AND CHARACTERIZATION OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR RECEPTOR-B: A NOVEL RECEPTOR FOR MEMBERS OF GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR FAMILY OF NEUROTROPHIC FACTORS

C.-Y. WANG,*§ J. NI,† H. JIANG,‖ T.-A. HSU,† M. DUGICH-DJORDJEVIC,§ L. FENG,§¶ M. ZHANG,† L. MEI,‡¶** R. GENTZ,†** and B. LU§**††

*Genetics Graduate Program George Washington University, Washington, DC 20052, U.S.A.

†Human Genome Sciences, Inc., Rockville, MD 20850-3338, U.S.A.

‡Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, VA 22908, U.S.A.

§Laboratory of Developmental Neurobiology and ‖Section on Growth Factors, NICHD, Bethesda, MD 20892-4480, U.S.A.

¶Shanghai Research Center of Life Sciences Chinese Academy of Sciences, Shanghai, China 200031

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Glial cell line-derived neurotrophic factor (GDNF)[14] is a potent neurotrophic factor with diverse biological functions.[3,8,10,16,19,20,22] Signal transduction of GDNF is mediated by binding to a glycosyl-phosphatidylinositol (GPI)-linked receptor GDNFR-α and activation of c-RET tyrosine kinase.[5,11,23,24] The recent discovery of a new GDNF homolog neurturin[13] raises the possibility that multiple receptors exist for the members in the GDNF family. Here we report isolation of the gene encoding a new receptor called GDNFR-β. Sequence analysis indicated that GDNFR-β is also a GPI-linked protein, with 47% identity to GDNFR-α. The GDNFR-β transcript was preferentially expressed in the brain, spleen and lung, but moderate levels of GDNFR-β mRNA were also found in kidney and the entire gastrointestinal track. *In situ* hybridization revealed high expression levels in the entorhinal cortex and olfactory bulb, followed by cortex, septum, inferior and superior colliculus, and zona inserta. A laminar pattern of expression was detected in layer III of the cortex. Treatment with GDNF of PC12 cells transfected with the GDNFR-β gene activated mitogen-activated protein kinase (MAPK) and elicited neurite outgrowth. GDNFR-α and GDNFR-β together form a new family of GPI-linked receptors for GDNF-like molecules.

Human GDNFR-α sequences[11,23] were used to search homologs in the expression sequence tags database established by Human Genome Science Inc. (HGS), using the BLAST and BLASTN algorithms.[6,17] Two overlapping clones were found to have translated sequences of more than 40% homologous to that of rat GDNFR-α. These clones covered an open reading frame of 458 amino acid residues without the 5′ starting codon. The missing sequences for the first 6 amino acids was obtained by rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR),[7] and the full length gene is named GDNFR-β. The deduced human GDNFR-β protein contains 464 amino acid residues and has a predicted molecular weight of approximately 51,000 (Fig. 1a). Sequence analysis indicated that GDNFR-β is 47% identical to human GDNFR-α at protein level (Fig. 1b). All of the structure features that were previously demonstrated to be essential for GDNFR-α function are preserved in GDNFR-β: i) a signal peptide for secretion at N-terminus;[9] ii) three potential N-glycosylation sites located in similar positions for both genes; iii) all of the 30 cysteine residues conserved between GDNFR-α and GDNFR-β; iv) a stretch of 17 hydrophobic amino

**Co-senior authors.

††To whom correspondence should be addressed.

Abbreviations: EST, expression sequence tags; GDNF, glial cell line-derived neurotrophic factor; GDNFR, glial cell line-derived neurotrophic factor receptor; GFP, green fluorescence protein; GPI, glycosyl-phosphatidylinositol; MAPK, mitogen-activated protein kinase.

a

MILANVFCLFFFLDETLRSLASPSSLQGPELHGWRPPVDCVRANELCAAESNC

SSRYRTLRQCLAGRDRNTMLANKECQAALEVLQESPLYDCRCKRGMKKELQ

CLQIYWSIHLGLTEGEEFYEASPYEPVTSRLSDIFRLASIFSGTGADPVVSAKSN

HCLDAAKACNLNDNCKKLRSSYISICNREISPTERCNRRKCHKALRQFFDRVP

SEYTYRMLFCSCQDQACAERRRQTILPSCSYEDKEKPNCLDLRGVCRTDHLC

RSRLADFHANCRASYQTVTSCPADNYQACLGSYAGMIGFDMTPNYVDSSPTG

IVVSPWCSCRGSGNMEEECEKFLRDFTENPCLRNAIQAFGNGTDVNVSPKGPS

FQATQAPRVEKTPSLPDDLSDSTSLGTSVITTCTSVQEQGLKANNSKELSMCFT

ELTTNIIPGSNKVIKPNSGPSRARPSAALTVLSVLMLKLAL

* * *

b

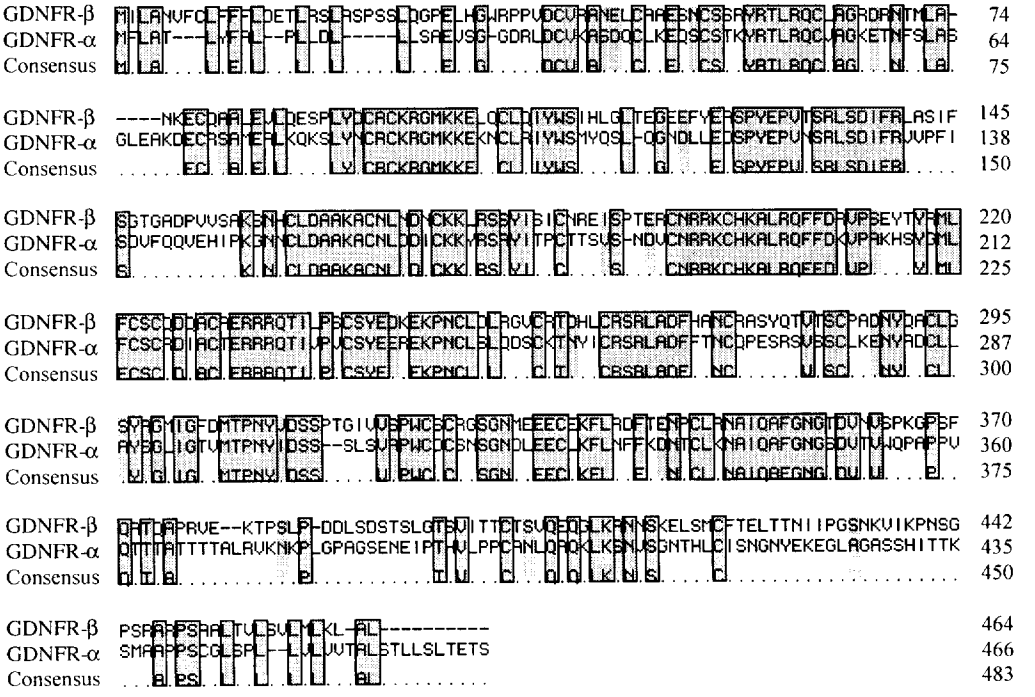

Fig. 1. Primary structure of GDNFR-β. (a) Amino acid sequence of human GDNFR-β. The 5′ signal peptide sequences are underlined by dotted line, and the putative cleavage site by arrow. N-glycosylation sites are boxed, and all the conserved cysteine residues are in bold letters. *: amino acid residues that constitute the GPI-linkage site. The double-underline indicates hydrophobic domain of the GPI attachment site. (b) Amino acid sequence comparison between human GDNFR-α and GDNFR-β. Boxes indicate identical amino acids. Conserved sequences are shown as shaded areas.

acid at the C-terminus preceded by a group of three small amino acids (Gly, Ser, Asn), which is a typical cleavage/binding site for a GPI linkage.[15] These results suggest that GDNFR-β is a member of a new receptor family for GDNF-like neurotrophic factors.

To study the tissue distribution of the gene, a 633 bp cDNA from rat was cloned by PCR (Primers: U1: 5′-GTCCGGGCCAATGAGCTGTGTGCC-3′. L1: 5′-TGTCGTGAG CTCTGTGAAGCACATG C-3′). This fragment is the rat homolog of human GDNFR-β (identity: DNA, 90%; amino acid, 94%) but not rat homolog of GDNFR-α (identity: DNA, 59%; amino acid, 44%). Northern blots using this cDNA probe detected a message at approximately

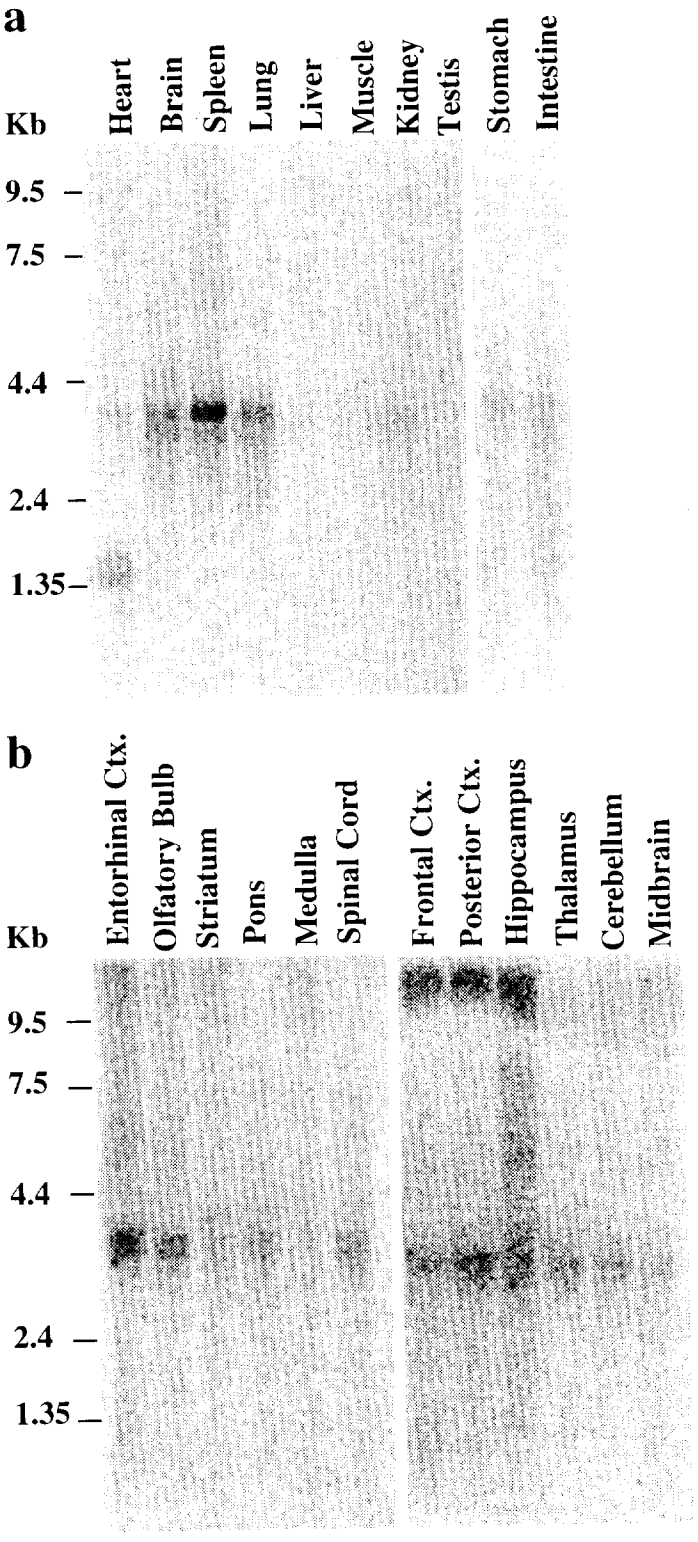

Fig. 2. Detection of rat GDNFR-β mRNA by northern blot. Nitrocellulose filters containing RNA from various rat tissues or brain areas (Origen, Inc. and Clontech) were hybridized with a 633 bp rat GDNFR-β cDNA probe ([α-^{32}P] dCTP nick translation, specific activity:10 cpm/μg^{9}) at high stringency for 2 h. The filter was then washed with 2 × SSC/0.05% SDS at room temperature for 40 min and with 0.1 × SSC/0.1% SDS at 50°C for 40 min, and exposed to Kodak XAR-5 films or PhosphoImager. All lanes in a blot contained equal amount of RNA, as evidenced by equal density of actin band (not shown). The DNA size markers are shown on the left of the blots. (a) The distribution of GDNFR-β mRNA in various adult tissues. Twenty five micrograms of total RNA was loaded in each lane. (b) Expression of GDNFR-β mRNA in different regions of the adult brain. Two micrograms of poly A(+) RNA was loaded in each lane.

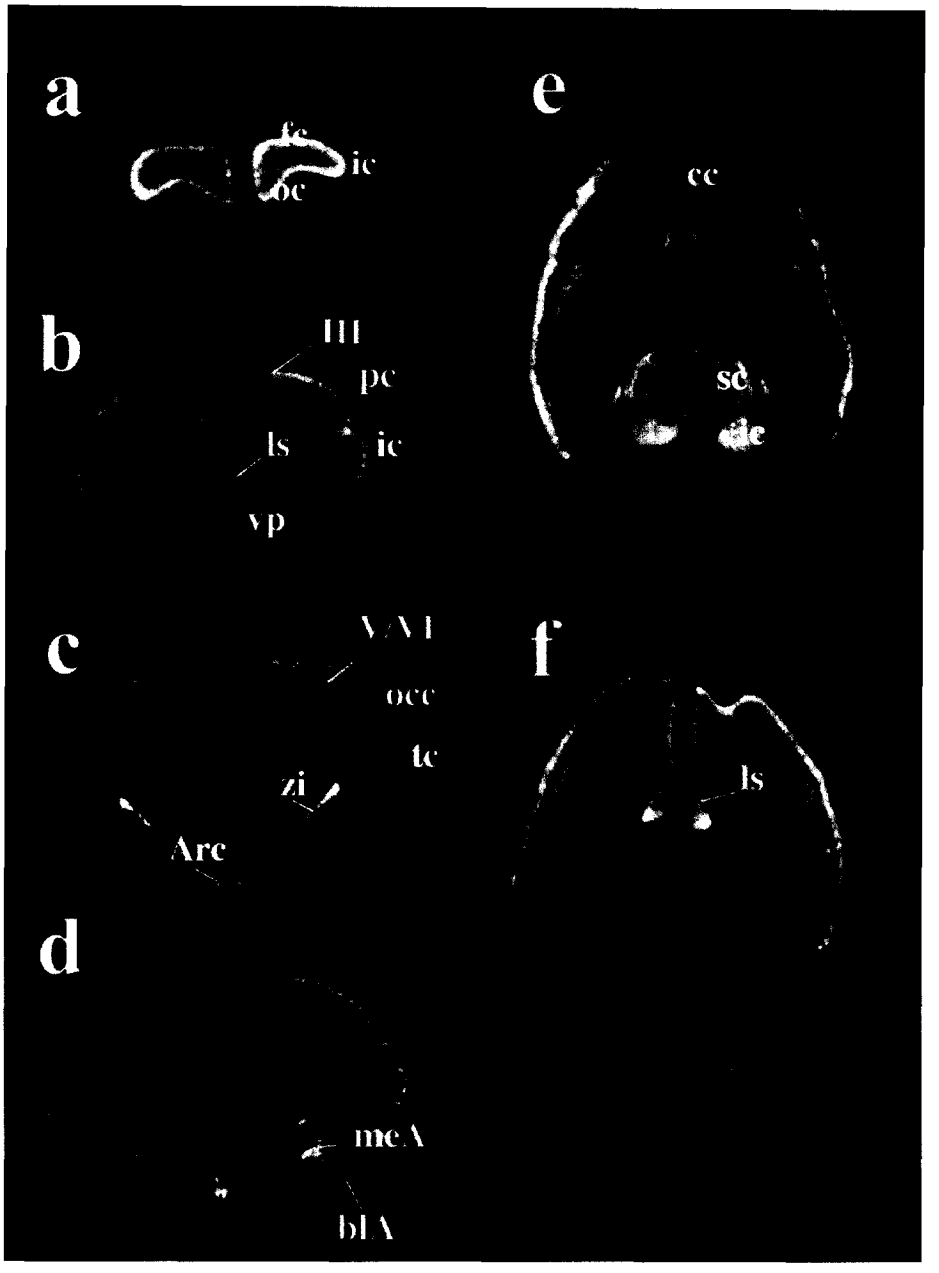

Fig. 3. Distribution of GDNFR-β mRNA in the adult rat brain as determined by *in situ* hybridization, using a standard protocol.[4] The coronal (a–d) and sagittal (e, f) sections from different levels of the brain were hybridized for 3 h at 50°C with an antisense [^{35}S] GDNFR-β cRNA, rinsed in 4 × SSC/20 mM DTT, followed by 30 min of RNase A (20 μg/ml) digestion at 37°C. The sections were then washed overnight in 2 × SSC at 25°C followed by 0.1 × SSC at 60°C for 1 h, dehydrated in a series of ethanol, air-dried and exposed to film. Non-specific labeling was determined in adjacent sections in each group hybridized with [^{35}S]-labeled sense probe or pretreated with 20 μg/ml RNase before probe hybridization. In sections at rostal regions of the brain (a) labeling was intense in layer III throughout the frontal cortex (fc), insular cortex (ic) and orbital cortex (oc). Sections at the level of the dorsal striatum (b) showed moderate labeling in the lateral septal nuclei (ls) and ventral palladium (vp) and diffuse labeling of layers V/VI throughout the parietal and insular cortices. Sections through the level of the hippocampus (c, d) demonstrated a continuation of intense signal in layer III and diffuse labeling of layers V/VI in the occipital cortex (occ) and temporal cortex (tc). The zona inserta (zi) and medial amygdaloid nucleus (meA) were intensely labeled while the arcuate nucleus (Arc) and basolateral amygdala (blA) exhibit moderate expression levels. Horizontal sections through the dorsal hippocampus (e) and ventral hippocampus (f) showed expression in the cingulate cortex (cc), inferior (ic) and superior (sc) colliculi.

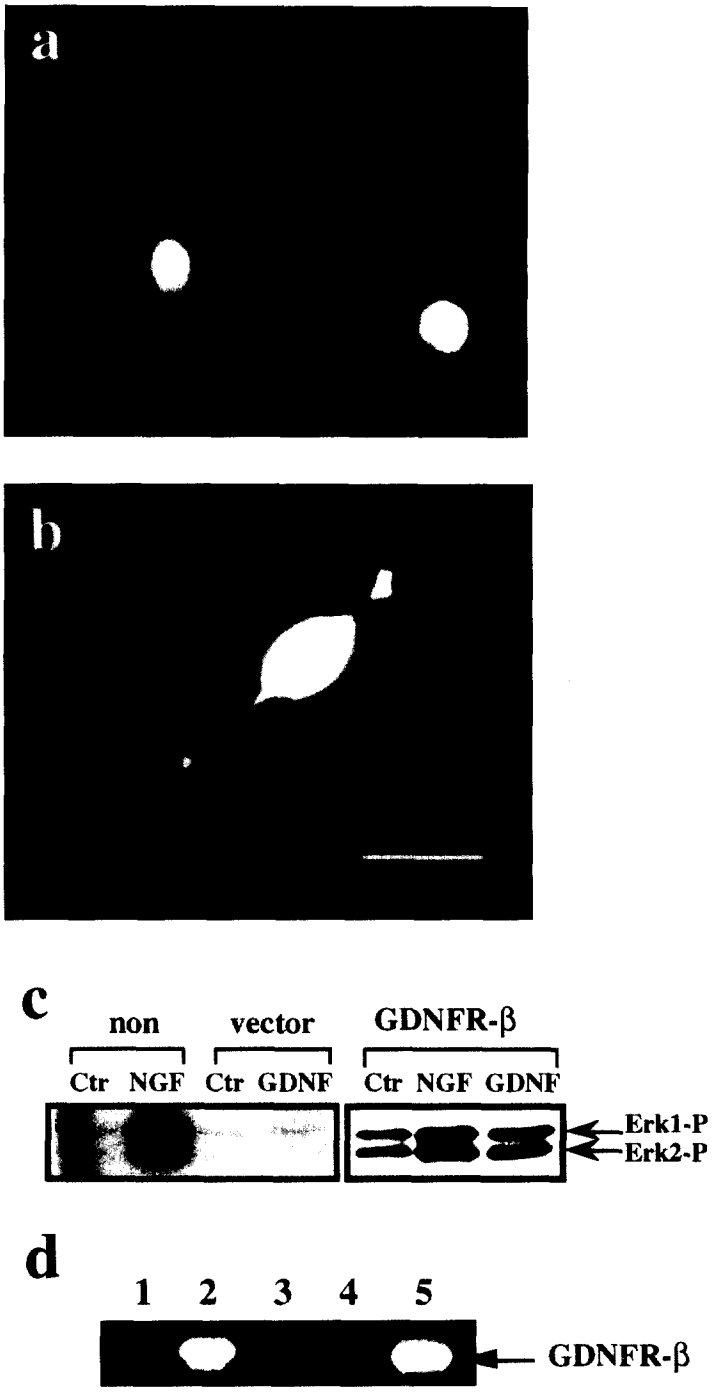

Fig. 4. Functional characterization of GDNFR-β in PC12 cells. (a, b) PC12 cells were transiently co-transfected with a GFP-expressing construct, together with either a mammalian expression vector (a) or the same vector inserted with the full length human GDNFR-β gene (b). Cells (10^5 cells/35 mm dishes) were then grown in serum free medium in the presence of GDNF (300 ng/ml) for two days. GFP (+) cells in approximately 30% of total dish area were examined, and two dishes were used for each condition. Neurite-bearing cells: neurites >3 × soma diameters. Scale bar=20 μm. (a) Two GFP (+) cells transfected with vector alone showing no neurites. (b) A GFP (+) cell transfected with GDNFR-β gene showing multiple neurites. (c) PC12 cells were either non-transfected (non), transfected with the vector alone (vector) or the vector containing GDNFR-β gene (GDNFR-β). Several GDNFR-β-expressing stable colonies were pooled. The cells were rinsed, exposed to NGF (25 ng/ml) or GDNF (300 ng/ml) for 30 min, and harvested. MAPK activation was measured by western blot using an antibody specific for activated MAPK. The amount of proteins loaded was the same for the first four lanes and last three lanes, respectively, since equal density of total MAPK bands were observed (not shown). Phosphorylated MAPKs are indicated as Erk1-P and Erk2-P. (d) RT-PCR to show that no GDNFR-β was found in parental PC12 cells but high level of GDNFR-β mRNA was detected in PC12 cells stably transfected with GDNFR-β gene. Templates used in RT-PCR: 1, none; 2, pC4-GDNFR-β plasmid; 3, cDNA derived from mRNA of parental PC12 cells; 4, none; 5, cDNAs derived from mRNAs of PC12 cells transfected with GDNFR-β gene.

3.6 kb in a variety of tissues of Sprague–Dawley rats (Fig. 2). The GDNFR-β mRNA was preferentially expressed in the brain, spleen and lung (Fig. 2a). Moderate to low levels of GDNFR-β mRNA were also detected in the kidney, stomach and small intestine, areas where GDNF plays a critical role.[22] There was no detectable GDNFR-β mRNA in skeletal muscle or liver. Within the brain, the highest levels of GDNFR-β mRNA were found throughout the cerebral cortex, particularly in the entorhinal cortex (Fig. 2b). Olfactory bulb and cerebellum also exhibited relatively high levels of expression. Spinal cord and midbrain, two areas containing neurons that are the target of GDNF regulation, expressed only moderate levels of the GDNFR-β gene (Fig. 2b).

Cellular localization of GDNFR-β mRNA was determined by *in situ* hybridization using a riboprobe derived from the same 633 bp construct. In the adult rat brain high levels of [^{35}S] GDNFR-β cRNA hybridization was localized to the cortex, lateral septal nuclei, bed nucleus of the stria terminalis, medial and basolateral amygdaloid nuclei, zona inserta, reticular thalamic nucleus, and the superior and inferior colliculus. Moderate labeling was evident in hypothalamic nuclei; notably the preoptic area, arcuate and the paraventricular hypothalamic nuclei. The most striking pattern of expression was in the cortex. A continuous band of hybridization was localized to cortical layer III extending from the cingulate at anterior levels to the perirhinal cortex and portions of the entorhinal cortex at more posterior levels. Layers V/VI displayed high levels of diffuse labeling with no obvious laminar pattern (Fig. 3).

To examine potential function of GDNFR-β, we transfected the full length human GDNFR-β gene into PC12 cells, together with the green fluorescence protein (GFP) gene to visualize GDNFR-β expressing cells. PC12 cells express low levels of endogenous RET tyrosine kinase, but neither GDNFR-α[23] nor GDNFR-β was detected (Fig. 4d). No neurites were found in untreated GFP(+) cells, but 90% of GFP(+) cells showed neurites after nerve growth factor treatment. Treatment with GDNF elicited few neurites in vector-transfected cells (1.8 ± 1.0%; Fig. 4a). By contrast, a substantial number of cells (17.6 ± 2.5%) transfected with GDNFR-β gene exhibited evident neurite outgrowth two days after treatment with 300 ng/ml GDNF (Fig. 4b). Lower concentrations of GDNF (25–100 ng/ml), however, were ineffective in promoting neurite outgrowth (data not shown).

GDNF has been shown to activate mitogen-activated protein kinase (MAPK) in neuroblastoma cells.[27,29] To determine whether GDNF is capable of signaling through GDNFR-β, PC12 cells were stably transfected with the GDNFR-β gene. Expression of GDNFR-β in the transfected cells was monitored by RT-PCR. As shown in Fig. 4d, transfected PC12 cells expressed high levels of GDNFR-β gene. MAPK activation was detected by an antibody specifically against the activated MAPK (Promega). Treatment with GDNF (300 ng/ml) for 30 min elicited a significant MAPK activation only in GDNFR-β transfected cells but not in control cells (Fig. 4c).

Based on the structural similarity between GDNFR-α and GDNFR-β, and biochemical (MAPK phosphorylation) and biological (neurite outgrowth) responses to GDNF of PC12 cells transfected with GDNFR-β gene, we suggest that GDNFR-β is a receptor for the GDNF family of neurotrophic factors. We have noticed that the concentration of GDNF (approximately 10 nM) required to activate MAP kinase and to induce neurite outgrowth in GDNFR-β transfected PC12 cells was much higher than that used to enhance the survival of dopaminergic neurons.[14] The dissociation constants (K_d) for GDNF binding to GDNFR-α have been shown to be in the range of 3–300 pM.[11,23] Thus, GDNF may not be the preferred ligand for GDNFR-β. During the preparation of this paper, four papers reported the cloning of GDNFR-β gene.[1,2,12,21] Two papers showed that neurturin is the ligand for GDNFR-β,[2,12] while the other two provided evidence that GDNFR-β can mediate both GDNF and neurturin signaling through c-RET tyrosine phosphorylation.[1,21] Thus, further investigation is required to determine whether neurturin is the preferred ligand for GDNFR-β.

Our study also identified several potentially new targets for GDNF family of proteins. Northern blot detected a high level in the spleen and lung. It is interesting to note that neurturin mRNA is also highly expressed in the lung.[13,26] *In situ* hybridization experiments showed that GDNFR-β mRNA is expressed in a striking laminar pattern in layers III and V/VI throughout the cortex. We have also detected strong signals in lateral septal nuclei. A recent report showed that GDNF infused into the lateral ventricle prevents the loss of septum cholinergic neurons induced by a unilateral transection of the fimbria–fornix.[28] However, high concentrations of exogenous GDNF were required to elicit the neurotrophic effects, and GDNF mRNA is non-detectable in either septum or hippocampus.[18,25] Analogous to the GDNF effects on PC12 cells transfected with GDNFR-β gene, it is possible that at such high concentrations, GDNF mimics other member(s) of the GDNF family and acting on GDNFR-β, rather than GDNFR-α. Finally, high levels of GDNFR-β message were also found in superior and inferior colliculus, areas that are involved in auditory and visual information processing. Thus, GDNFR-β may function as a receptor distinct from the GDNF/GDNFR-α signaling system in certain regions of the brain.

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