Mitogenic effect of glial cell line-derived neurotrophic factor is dependent on the activation of p70 S6 kinase but independent of the activation of ERK and up-regulation of Ret in SH-SY5Y cells

Yoko Hirata and Kazutoshi Kiuchi

Department of Biomolecular Science, Faculty of Engineering, Gifu University, Gifu 501-1193 and the Laboratory for Genes of Motor Systems, Bio-Mimetic Control Research Center, RIKEN, Nagoya 463-0003, Japan

The number of text pages of the whole manuscript: 32
The number of figures: 7

Corresponding author:
Dr. Yoko Hirata
Department of Biomolecular Science, Faculty of Engineering, Gifu University
1-1 Yanagido, Gifu 501-1193, Japan
Phone:+81-58-293-2609
Fax:+81-58-230-1893
email:yoko@biomol.gifu-u.ac.jp

Acknowledgements:

This work was supported by the Frontier Research Program, RIKEN.

Abstract

Glial cell line-derived neurotrophic factor (GDNF) activates c-Ret tyrosine kinase and several downstream intracellular pathways; the biological effects caused by the activation of each of these pathways, however, remain to be elucidated. Here we report the ability of GDNF to induce proliferation, rather than differentiation, of neuroblastoma cells (SH-SY5Y) via targeting the signaling pathway responsible for mediating this proliferative effect. GDNF induces the phosphorylation of Akt and p70S6 kinase (p70S6K) in SH-SY5Y cells in which Ret protein expression is relatively low. Interestingly, treating SH-SY5Y cells with retinoic acid greatly increases Ret protein levels and GDNF-induced Ret tyrosine phosphorylation, but does not affect the mitogenic action of GDNF and the activation of the Akt/p70S6K pathway. In contrast, the activation of the ERK pathway and the resulting induction of immediate-early genes parallel the increases in Ret protein levels. Rapamycin, a specific inhibitor of p70S6K activation by mammalian target of rapamycin, completely prevents GDNF-induced proliferation and activation of p70S6K. These results suggest that GDNF promotes cell proliferation via the activation of p70S6K, independent of the ERK signaling pathway, and that GDNF activates the Akt/p70S6K pathway more efficiently than the ERK pathway in the cells in which Ret expression is low.

Theme: Development and regeneration

Topic: Neurotrophic factors: receptors and cellular mechanisms

Keywords: ERK, GDNF, Ret, p70S6K, SH-SY5Y

1. Introduction

Glial cell line-derived neurotrophic factor (GDNF), a distant member of the transforming growth factor-β superfamily, was characterized as a potent survival factor for midbrain dopaminergic neurons [26]. Subsequent studies indicate that GDNF exhibits a variety of other biological activities and has effects on several types of neurons including motor neurons [19], as well as sensory and autonomic neurons [8]. GDNF also prevents the axotomy-induced death of facial or spinal cord motorneurons [19,29,47], and the degeneration of mesencephalic dopaminergic neurons following axotomy [3] and chemical lesions by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [37] or 6-hydroxydopamine [23]. GDNF exerts its effects via a multicomponent receptor complex comprised of the c-Ret receptor tyrosine kinase and the glycosylphosphatidylinositol (GPI)-anchored receptor, GFRα1, which is required for ligand binding [24,38]. GDNF activates the c-Ret kinase and downstream signaling pathways, including the Ras/extracellular signal-regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways [43,44]. In addition to Ret-dependent pathways, GDNF can also signal cells lacking the Ret receptor via a mechanism that involves binding to GFRα1 receptors, stimulation of a cytoplasmic GFRα1-associated Src-like kinase, and activation of the cyclic AMP response element binding protein (CREB) [41].

The GDNF-induced activation of the PI3K pathway has been implicated in lamellipodia

formation, which is required for neuritogenesis in neuroepithelioma cells [43], and in the survival of spinal cord motoneurons [36]. The PI3K pathway was also shown to be involved in the GDNF-induced differentiation of dopaminergic neurons [32] and in the GDNF-induced proliferation of enteric neuroblasts [15].

In this paper, we demonstrate that a mitogenic effect of GDNF on SH-SY5Y cells in which Ret and GFRα1 are endogenously expressed is dependent on the activation of p70 S6 kinase (p70S6K) but independent of the activation of ERK. In addition, the up-regulation of Ret does not have a relevant contribution to the mitogenic action and the activation of p70S6K induced by GDNF. The SH-SY5Y model allowed us to study GDNF-induced Akt/p70S6K signaling without the influence of the ERK pathway. Some of these data have been published previously in abstract form [21,22].

2. Materials and methods

2.1. Materials

Anti-phosphotyrosine antibody (Ab) 4G10 and anti-Rsk2 Ab were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-p70S6K, anti-c-Fos (4), anti-Egr-1 (C-19) and anti-Ret Abs, C-19 (specific for Ret short isoform) and C-20 (specific for Ret long isoform) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GFRα1 Ab was from

Pharmingen (San Diego, CA). Phospho-specific Abs for ERK, Rsk, CREB, Akt and p70S6K, anti-ERK Ab and anti-CREB Ab were from New England Biolabs, Inc. (Beverly, MA). Anti-actin (Ab-1) Ab was from Calbiochem-Novabiochem Corporation (San Diego, CA). GDNF was obtained from Almone Labs Ltd. (Jerusalem, Israel).

2.2. Cell cultures

The human neuroblastoma cell line SH-SY5Y [5,6], the human neuroepithelioma cell line SK-N-MC [5,6], and the mouse neuroblastoma cell line Neuro2a (ATCC #CCL 131) were cultured in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% heat-inactivated fetal bovine serum (Biofluids, Inc., Rockville, MD) at 37°C in 5% CO_2. The rat pheochromocytoma cell line PC12 [16] was grown in DMEM supplemented with 7% horse serum (BioWhittaker, Inc., Walkersville, MD) and 4% fetal bovine serum at 37°C in 5% CO_2.

2.3. Western blotting

Cells were lysed in SDS-Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol) and sonicated for ~ 20 s. Total cell lysates (40 µg protein) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was performed with the appropriate antibody, and immunoreactivity was visualized with the enhanced chemiluminescence (ECL) system (Amersham Biosciences Corp., Piscataway, NJ). In some

cases, blots were reprobed with different antibodies after stripping in 62.5 mM Tris-HCl (pH 6.7)/100 mM β-mercaptoethanol/2% SDS at 55°C for 30 min.

2.4. Cell proliferation assay

Cell proliferation was determined with 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) using a Cell Counting Kit (Wako Pure Chemical Industries, Osaka, Japan). Briefly, WST-1 was added to the culture at a final concentration of 500 nM. After incubation for 2-4 h at 37°C, the absorbance was measured photometrically at 405 nm using a reference wavelength of 690 nm. Statistical differences between controls and treated groups were assessed by the two-tailed, unpaired Student's t-test.

2.5. Immunoprecipitation

Cells were lysed for 10 min on ice in 10 volumes of the lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 μM okadaic acid, 1 mM sodium vanadate and 100 mM β-glycerophosphate, after which the lysates were centrifuged at 12,000 x g for 10 min. The lysates were immunoprecipitated by incubating with an appropriate Ab at 4°C overnight followed by incubation with 40 μl of a 1:1 slurry of Protein A/G+ agarose at 4°C for 2 h. The immunocomplexes bound to the agarose were washed 3 times with 1 ml of the lysis buffer and once with 1 ml of 50 mM Tris-HCl (pH

6.8), and eluted with 40 µl of SDS-Laemmli sample buffer containing 5% β-mercaptoethanol. The samples were then boiled for 5 min, and 20 µl aliquots of eluate were subjected to SDS-PAGE and immunoblotted as described above.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using a RNeasy Total RNA kit (Qiagen, Hilden, Germany). Single strand cDNA was synthesized from 1 µg total RNA in a volume of 20 µl containing 0.5 µg oligo$(dT)_{12\text{-}18}$ primer (Invitrogen Corporation, Carlsbad, CA), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 U RNasin ribonuclease inhibitor (Promega Corporation, Madison, WI), 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 10 mM DTT and 200 U MMLV reverse transcriptase (SuperScript™ II, Invitrogen Corporation). Reactions were incubated for 50 min at 42°C, then terminated by heating for 15 min at 70°C. To remove RNA complementary to the cDNA, 2 U of RNase H were added and the mixture was incubated for 20 min at 37°C. PCR was performed using the following primers: ret, F:5'-GCTGAGGGACGGAAGATGAAG-3' and R:5'-CAGTTGTCTGGCCTCTCCATCC-3' giving a 281-bp product (30 cycles); GFRα1, F:5'-GCACAGCTACGGGATGCTCTTCTG-3' (mouse, rat), F:5'-GCACAGCTACGGAATGC-TCTTCTG-3' (human) and R:5'-GTAGTTAG-GAGTCATGACTGTGCCAATC-3' (mouse, rat), R:5'-GTAGTTGGGGGTCATGACTGTG-CCAATA-3' (human) giving a 286-bp product [2] (35 cycles); GAPDH (glyceraldehyde-3-

phosphate dehydrogenase), F:5'-GGAGATTGTTGCCATCAACGAC-3' (mouse, rat), F:5'-GGATATTGTTGCCATCAATGAC-3' (human) and R:5'-ATGAGCCCTT-CCACAATGCCAAAG-3' (mouse, rat), R:5'-ATGAGTCCTTCCACGATACCAAAG-3' (human) giving a 441-bp product [25] (25 cycles).

3. Results

3.1 Effects of GDNF on SH-SY5Y cells

First, to determine the biological effects of GDNF on the human neuroblastoma cell line SH-SY5Y, SH-SY5Y cells were grown in serum-containing medium in the presence or absence of GDNF. GDNF significantly increased the number of dividing cells in a concentration-dependent manner (Fig. 1, Fig. 2A, B). Whereas GDNF elicits cell differentiation of Purkinje cells and the neuroblastoma cell line, TGW-I-nu [27,28], phase-contrast microscopy revealed no apparent morphological changes in the SH-SY5Y cells (Fig. 2B) under these conditions. These results suggest that GDNF alone acts as a mitogenic factor, rather than a differentiating factor, in SH-SY5Y cells.

3.2. Effect of GDNF on Neuro2a, SK-N-MC and PC12 cells

To investigate whether the mitogenic action of GDNF is observed in different cell lines that express either Ret or GFRα1, SK-N-MC, Neuro2a and PC12 cells were cultured in the

presence of GDNF. SK-N-MC, a human neuroepithelioma cell line, expresses GFRα1, but not Ret [43]. Transfection of Ret into SK-N-MC cells renders these cells responsive to GDNF, an action that is not observed in the parental cell line. This finding indicates that SK-N-MC has intrinsic functional GFRα1 receptors, but not Ret receptors. GDNF did not affect the proliferation of SK-N-MC cells at concentrations of 10 to 100 ng/ml (Fig. 3). Neuro2a, a mouse neuroblastoma cell line, endogenously expresses Ret, but does not express detectable levels of GFRα1 mRNA [24]. Neuro2a also did not respond to GDNF at concentrations of 10 to 100 ng/ml (Fig. 3). Similarly, PC12, a rat pheochromocytoma cell line, endogenously expresses Ret, but does not express detectable levels of GFRα1 [11]. As with the Neuro2a cells, PC12 cells did not respond to GDNF (Fig. 3). These results indicate that the mitogenic action of GDNF requires the expression of both Ret and GFRα1 proteins.

3.3. RA stimulates the level of Ret protein but does not affect the action of GDNF

The expression of Ret and GFRα1 proteins in SH-SY5Y cells was confirmed with Western blotting. Although the expression was relatively low in these cells, Ret was detected as a doublet comprised of 150 kDa and/or 170 kDa glycoproteins of both short (1072 amino acids) and long isoforms (1114 amino acids) (Fig. 2C). Ret protein expression in SH-SY5Y cells was enhanced by treating the cells with retinoic acid (RA), a compound demonstrated to induce ret mRNA in SH-SY5Y cells [9]. Indeed, RA strongly induced protein levels of 150 kDa Ret and

170 kDa Ret of both isoforms (Fig. 2C). Protein induction lasted at least 5 days. To obtain maximum induction of Ret, we usually pretreated the cells with 1-1.5 μM RA for 1 day. The expression of GFRα1 protein, in contrast, was not influenced by the RA treatment. RA treatment may have multiple effects on a number of cell pathways in addition to modulate Ret protein levels, however, at the least protein levels of several signaling molecules such as Gab1, Gab2, Grab2 and Shc were not affected under the experimental conditions (data not shown). Although we observed neuritic extensions in SH-SY5Y cells treated with RA, as previously reported [31], both untreated and RA-treated cells exhibited increased proliferation in response to GDNF (Fig. 2A, B). It is surprising that RA-induced overexpression of Ret does not appear to contribute to the mitogenic effects of GDNF. GDNF treatment for 3 days resulted in a slight increase in Ret protein level; this increase, however, was less than that in RA-treated cells (data not shown). These results exclude the possibility that the mitogenic action of GDNF alone is due to the induction of Ret.

To confirm that induced Ret in RA-treated SH-SY5Y cells responds to GDNF, the cells were treated with GDNF followed by immunoprecipitation with antibodies that recognize short and long forms of Ret. The immunoprecipitated Ret proteins were then assayed for ligand-induced tyrosine phosphorylation by probing with anti-phosphotyrosine antibody. GDNF stimulated the tyrosine phosphorylation of p170 Ret, a form located in the plasma

membrane, (Fig. 2D). p150 Ret, an incompletely processed form of Ret present in the endoplasmic reticulum, was not phosphorylated. Moreover, even in the absence of ligand, Ret showed a high constitutive level of tyrosine phosphorylation in RA-treated cells similar to that exhibited by COS cells that overexpress Ret [40]. In contrast, in naive SH-SY5Y cells, GDNF-induced tyrosine phosphorylation of Ret was very minimal under these conditions. It may be possible that this low level of tyrosine phosphorylation is sufficient to mitogenic signal.

3.4. Effects of GDNF on various signaling pathways

To investigate the initial event associated with the mitogenic action of GDNF, various types of cells were treated with GDNF for 10 min, then the phosphorylation of signaling molecules was examined by Western blotting (Fig. 4A, B). GDNF stimulates the PI3K/Akt and ERK pathways via activation of Ret tyrosine kinase in several contexts [4,15,32,36,41]. The phosphorylation status of downstream PI3K targets, Akt and p70S6K, was studied by immunoblotting to determine their activation state (Fig. 4A). In untreated SH-SY5Y cells and RA-treated SH-SY5Y cells, GDNF treatment stimulated the phosphorylation of Akt and p70S6K. The phosphorylation of p70S6K was also observed as a reduction in mobility upon SDS-PAGE. The extent of GDNF-induced phosphorylation of Akt and p70S6K was similar in RA-treated SH-SY5Y cells and in untreated SH-SY5Y cells, regardless of the amount of Ret

expressed. In contrast, the phosphorylation of p44/p42 ERK was much more evident in RA-treated SH-SY5Y cells compared to that in untreated SH-SY5Y cells (Fig. 4B). Akt, p70S6K, and ERK protein levels were not significantly affected by GDNF and RA treatment. These results indicate that the activation of Akt and p70S6K appears to be correlated with the mitogenic action of GDNF, but not with the expression of Ret protein. On the other hand, the phosphorylation of ERKs was related to Ret protein levels. These results suggest that the Akt/p70S6K pathway and the ERK pathway are independently regulated by the activation of the GDNF receptor complex. There was no obvious stimulation of Akt, p70S6K, and ERKs in SK-N-MC cells, and little activation of these pathways in Neuro2a and PC12 cells (Fig. 4A, B). Moreover, GDNF did not promote proliferation in these 3 cell lines. These results further support that both Ret and GFRα1 are necessary to stimulate these signaling molecules.

The expression of Ret and GFRα1 in these cell lines was confirmed by assessing both the mRNAs and proteins of these receptors using RT-PCR and Western blotting. As shown in Fig. 4C and D, both ret mRNA and Ret protein were expressed in SH-SY5Y cells, Neuro2a cells and PC12 cells, but not in SK-N-MC cells. GFRα1 mRNA and protein were expressed in SH-SY5Y cells and SK-N-MC cells. Faint GFRα1 mRNA signals were detected in PC12 cells and Neuro2a cells under these experimental conditions. GFRα1 protein, however, was not detectable in these cell lines. The observation that mRNA levels does not always parallel

protein levels was also evident in RA-treated SH-SY5Y cells, in which the induction of GFRα1 mRNA was obvious, but GFRα1 protein level was unchanged.

3.5. Similar time course of GDNF-induced signaling in naive SH-SY5Y cells and in RA-treated SH-SY5Y cells

The GDNF-induced stimulation of the Akt/p70S6K and ERK pathways was further characterized by examining the time course of GDNF action on these pathways in untreated or naive and RA-treated SH-SY5Y cells. ERK and p70S6K phosphorylation was elevated 30 min after GDNF treatment; this elevation lasted up to 12 h after stimulation with GDNF and gradually decreased with time (Fig. 5A). The time course of both ERK and p70S6K phosphorylation was similar in naive and RA-treated SH-SY5Y cells.

Activation of the ERK signaling pathway triggers the induction of specific immediate-early gene expression [39]. In RA-treated SH-SY5Y cells, GDNF treatment elicited a rapid increase in c-Fos and Egr-1/NGFI-A/Zif268 protein expression that peaked 2 h after treatment (Fig. 5B). There was a slight induction of these immediate-early genes in naive SH-SY5Y cells. Reprobing the transfer membrane or blot with an anti-actin antibody showed that protein loading in the different lanes was similar. Taken together, these results suggest that GDNF induces the expression of c-Fos and Egr-1/NGFI-A/Zif268 through the ERK signaling pathway, but not through the PI3K/Akt kinase signaling pathway.

The 90 kDa ribosomal S6 kinase-2 (Rsk2), the first CREB kinase shown to be induced by a neurotrophin, is a downstream target of the Ras/ERK pathway [46]. Therefore, it would be reasonable to examine whether GDNF induces the phosphorylation of Rsk and CREB. As shown in Fig. 5C, GDNF stimulated the phosphorylation of Rsk and CREB in a time course similar to that of p44/42 ERK. These results suggest that GDNF stimulates the phosphorylation of CREB via activation of Rsk2, which is required for growth factor-stimulated transcription of the c-Fos gene [7].

3.6. Inhibition of p70 S6 kinase but not ERK kinase reverses GDNF-induced proliferation

Cells were treated with rapamycin to determine the role of the Akt/p70S6K and ERK kinase pathways in mediating the mitogenic effects of GDNF. As shown in Fig. 6A, rapamycin completely blocked both GDNF-induced and RA-, GDNF-induced cell proliferation. In contrast, PD98059, an inhibitor of the ERK kinase, had no effect on mitogenic action of GDNF (Fig. 6B). Western analysis confirmed that rapamycin specifically prevented the phosphorylation of p70S6K (Fig. 6C). The phosphorylation of Akt and ERK were not affected by rapamycin. LY294002, a PI3K inhibitor, blocked GDNF-induced proliferation of SH-SY5Y cells and GDNF-induced phosphorylation of Akt and p70S6K but not ERK, confirming that Akt and p70S6K are downstream of PI3K (data not shown). These

results clearly indicate that the activation of p70S6K is responsible for the mitogenic action of GDNF, whereas ERKs are not involved in this signaling pathway.

4. Discussion

The mitogenic action of GDNF has been mainly described in mammalian enteric nervous system progenitors. GDNF increases the uptake of BrdU from neuronal crest cells immuno-isolated from the gut of quail or rat embryo [18,45]. While GDNF and neurturin, another member of the GDNF family, greatly increase the number of BrdU/Ret double-labeled enteric neuron precursors, removal of GDNF or neurturin from the culture medium decreases cell proliferation [20]. Along with the observation that the death of cultured Ret-expressing cells increases in the absence of growth factor, these studies suggest that GDNF and neurturin are capable of promoting both proliferation and survival of enteric neuron precursors. We also see increases in the number of SH-SY5Y neuroblastoma cells, similar to those increases observed for enteric neuron precursors. However, GDNF does not affect the survival of SH-SY5Y cells, since these cells grow normally in serum-containing media void of GDNF. In SH-SY5Y cells, therefore, we can only study the proliferative effects of GDNF and its effects on downstream pathways.

Although the intracellular pathways involving oncogenic forms of Ret have been

extensively studied [1,10,13,34], less is known about the signaling mechanisms involving endogenous Ret in neuronal cells. We identified specific signaling pathways responsible for mediating the mitogenic actions of GDNF. In naive SH-SY5Y cells, GDNF activates mainly the PI3K pathway. Observation that p70S6K is a critical signaling molecule responsible for mediating the mitogenic effect of GDNF in SH-SY5Y neuroblastoma cells is in line with the generally accepted understanding of the role this kinase plays in regulating cell growth and proliferation [42]. The ERK kinase pathway, on the other hand, is minimally activated by GDNF and does not appear to contribute to GDNF-associated mitogenicity. These results agree with and extend the findings of other groups. In enteric crest-derived cells, GDNF activates both the PI3K and MAP kinase pathways, however, the latter is not required for proliferation of these cells [15]. Furthermore, several experiments demonstrated that the PI3K pathway is involved in cell survival and differentiation. A PI3K inhibitor LY294002, but not an ERK kinase (MEK) inhibitor PD98059, prevents GDNF family ligand-mediated neuronal survival [4,14,36]. The inhibition of PI3K blocks cellular differentiation mediated by GDNF in dopaminergic neurons [32] and Ret-induced lamellipodia formation [43]. These diverse findings indicate that the multiple functions of GDNF are mediated by the PI3K pathway, and the response to GDNF depends mainly on the cellular context.

In contrast, in RA-treated SH-SY5Y cells, GDNF activates both the PI3K and ERK

pathways. The GDNF-associated activation of the ERK signaling pathway results in the phosphorylation of Rsk and its downstream target, CREB, and in the induction of immediate-early genes, such as c-fos and Erg1/NGFI-A/Zif268. Because the expression of immediate-early genes is known to be regulated by the ERK pathway [39], our observation strongly suggests that the GDNF-induced expression of these immediate-early gene products in SH-SY5Y cells is regulated by the ERK pathway, not the PI3K pathway, and that these genes are unnecessary for the GDNF-induced proliferation of SH-SY5Y cells. Nonetheless, in certain cell types, the ERK pathway may play a role in the control of cell growth. There is direct evidence that ERKs have an effector function in the control of cell proliferation. In experiments using interfering mutants of ERK mutants, Pages and colleagues showed that ERKs are necessary for fibroblast proliferation [30]. On the other hand, a variety of experiments indicate that a primary function of the ERK signaling pathway is to induce differentiation [35]. For example, activation of MAP kinase kinase (MEK) is sufficient to induce neurite outgrowth in PC12 cells [12]. Our observations that RA-treated SH-SY5Y cells have longer neurite extensions compared to those of naive SH-SY5Y cells, reveal the difficulty of assessing the effects of GDNF on differentiation. Further studies are required, therefore, to determine the role of the ERK signaling pathway in cell differentiation in RA-treated SH-SY5Y cells.

Treatment of SH-SY5Y cells with retinoic acid results in increased p170 Ret expression and a basal level of tyrosine phosphorylation that clearly exceeds GDNF-stimulated tyrosine phosphorylation of this receptor in naive cultures. In spite of this observation, enhanced signaling through Akt/p70S6K is not detected nor is there an increase in cellular replication rate. This ligand-independent tyrosine phosphorylation of Ret may not cause the phosphorylation of tyrosine residues such as tyrosine 1062 important for the intracellular signaling downstream of Ret [17]. Alternatively, it could be due to cryptic localization of increased Ret protein, compartmentalized in such a way as to access the ERK cascade, but not PI3K pathway.

Experiments performed with SK-N-MC cells showed that the activation of the Akt/p70S6K pathway requires Ret expression. In SHEP neuroblastoma cells and in Ret-/- DRG neurons, Poteryaev et al. [33] showed that GDNF induces Ret-independent MAPK signaling via GFRα1. While we did not observe this type of Ret-independent signaling in our SK-N-MC cell preparations, Ret-independent signaling has been described in RN33B cells, a raphe nucleus cell line that expresses high levels of GFRα1, but no Ret [41]. One explanation for these apparent discrepancies for the role of Ret in GDNF-associated signaling is that GFRα1 expression may have to reach a certain threshold level in order for the GDNF signal to be transmitted independent of Ret [41].

Given this hypothesis, it is surprising that the levels of GDNF-induced Ret phosphorylation we observed in our SH-SY5Y cell preparations were sufficient to activate the Akt/p70S6K pathway. The simplest explanation for this observation is that GDNF transmits its signal preferentially and more efficiently to the Akt/p70S6K pathway, rather than to the ERK pathway. Alternatively, a unique signaling pathway that is yet to be identified may be involved in this phenomenon. The mechanism underlying this phenomenon is currently under investigation in our laboratory.

In summary, we characterized the signaling pathways underlying the mitogenic effects of GDNF on neuroblastoma cells (Fig. 7). GDNF promotes cell proliferation via the activation of Akt/p70S6 kinase, independent of the ERK signaling pathway. The level of Ret expression required for the activation of the Akt/p70S6K pathway is very different from that required for the activation of the ERK pathway. SH-SY5Y and RA-treated SH-SY5Y cell models will facilitate further investigation of GDNF signaling that is involved in the proliferation and differentiation of these cells.

References

[1] N. Asai, T. Iwashita, M. Matsuyama, M. Takahashi, Mechanism of activation of the ret proto-oncogene by multiple endocrine neoplasia 2A mutations, Mol. Cell. Biol. 15 (1995)

1613-1619.

[2] R.H. Baloh, M.G. Tansey, J.P. Golden, D.J. Creedon, R.O. Heuckeroth, C.L. Keck, D.B. Zimonjic, N.C. Popescu, E.M. Johnson Jr., J. Milbrandt, TrnR2, a novel receptor that mediates neurturin and GDNF signaling through ret, Neuron 18 (1997) 793-802.

[3] K.D. Beck, J. Valverde, T. Alexi, K. Poulsen, B. Moffat, R.A. Vandlen, A. Rosenthal, F. Hefti, Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain, Nature 373 (1995) 339-341.

[4] V. Besset, R.P. Scott, C.F. Ibanez, Signaling complexes and protein-protein interactions involved in the activation of the Ras and phosphatidylinositol 3-kinase pathways by the c-Ret receptor tyrosine kinase, J. Biol. Chem. 275 (2000) 39159-39166.

[5] J.L. Biedler, L. Helson, B.A. Spengler, Morphology on growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture, Cancer Res. 33 (1973) 2643-2652.

[6] J.L. Biedler, S. Roffler-Tarlov, M. Schachner, L.S. Freedman, Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones, Cancer Res. 38 (1976) 3751-3757.

[7] J.C. Bruning, J.A. Gillette, Y. Zhao, C. Bjorbaeck, J. Kotzka, B. Knebel, H. Avci, B. Hanstein, P. Lingohr, D.E. Moller, W. Krone, C.R. Kahn, D. Muller-Wieland, Ribosomal

subunit kinase-2 is required for growth factor-stimulated transcription of the c-Fos gene, Proc. Natl. Acad. Sci. USA 97 (2000) 2462-2467.

[8] A. Buj-Bello, V.L. Buchman, A. Horton, A. Rosenthal, A.M. Davies, GDNF is an age-specific survival factor for sensory and autonomic neurons, Neuron 15 (1995) 821-828.

[9] G. Bunone, M.G. Borrello, R. Picetti, I. Bongarzone, F.A. Peverali, V. de Franciscis, G.D. Valle, M.A. Pierotti, Induction of RET proto-oncogene expression in neuroblastoma cells precedes neuronal differentiation and is not mediated by protein synthesis, Exp. Cell Res. 217 (1995) 92-99.

[10] D. Califano, A. D'Alessio, G.L. Colucci-D'Amato, G. de Vita, C. Monaco, G. Santelli, P.P. di Fiore, G. Vecchio, A. Fusco, M. Santoro, V. de Franciscis, A potential pathogenetic mechanism for multiple endocrine neoplasia type 2 syndromes involves ret-induced impairment of terminal differentiation of neuroepithelial cells, Proc. Natl. Acad. Sci. USA 93 (1996) 7933-7937.

[11] M. Chiariello, R. Visconti, F. Carlomagno, R.M. Melillo, C. Bucci, V. de Franciscis, G.M. Fox, S. Jing, O.A. Coso, J.S. Gutkind, A. Fusco, M. Santoro, Signalling of the Ret receptor tyrosine kinase through the c-Jun NH_2-terminal protein kinases (JNKs): Evidence for a divergence of the ERKs and JNKs pathways induced by Ret, Oncogene 16 (1998) 2435-2445.

[12] S. Cowley, H. Paterson, P. Kemp, C.J. Marshall, Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells, Cell 77 (1994) 841-852.

[13] K. Durick, R.–Y. Wu, G.N. Gill, S.S. Taylor, Mitogenic signaling by Ret/ptc2 requires association with Enigma via a LIM domain, J. Biol. Chem. 271 (1996) 12691-12694.

[14] M. Encinas, M.G. Tansey, B.A. Tsui-Pierchala, J.X. Comella, J. Milbrandt, E.M. Johnson Jr., c-Src is required for glial cell line-derived neurotrophic factor (GDNF) family ligand-mediated neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway, J. Neurosci. 21 (2001) 1464-1472.

[15] P.J. Focke, C.A. Schiltz, S.E. Jones, J. J. Watters, M.L. Epstein, Enteric neuroblasts require the phosphatidylinositol 3-kinase pathway for GDNF-stimulated proliferation, J. Neurobiol. 47 (2001) 306-317.

[16] L.A. Greene, A.S. Tischler, Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor, Proc. Natl. Acad. Sci. USA 73 (1976) 2424-2428.

[17] H. Hayashi, M. Ichihara, T. Iwashita, H. Murakami, Y. Shimono, K. Kawai, K. Kurokawa, Y. Murakumo, T. Imai, H. Funahashi, A. Nakao, M. Takahashi, Characterization of

intracellular signals via tyrosine 1062 in RET activated by glial cell line-derived neurotrophic factor, Oncogene 19 (2000) 4469-4475.

[18] C.J. Hearn, M. Murphy, D. Newgreen, GDNF and ET-3 differentially modulate the numbers of avian enteric neural crest cells and enteric neurons in vitro, Dev. Biol. 197 (1998) 93-105.

[19] C.E. Henderson, H.S. Phillips, R.A. Pollock, A.M. Davies, C. Lemeulle, M. Armanini, L.C. Simpson, B. Moffet, R.A. Vandlen, V.E. Koliatsos, A. Rosenthal, GDNF: A potent survival factor for motoneurons present in peripheral nerve and muscle, Science 266 (1994) 1062-1064.

[20] R.O. Heuckeroth, P.A. Lampe, E.M. Johnson Jr., J. Milbrandt, Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro, Dev. Biol. 200 (1998) 116-129.

[21] Y. Hirata, H. Xiao, K. Kiuchi, Mitogenic action of GDNF dependent on Akt-p70 S6 kinase pathway, Soc. Neurosci. Abstr. 26 (2000) 1093.

[22] Y. Hirata, K. Kiuchi, Involvement of p70S6 kinase in the mitogenic action of GDNF, Neurochem. Res. 26 (2001) 1368.

[23] B.J. Hoffer, A. Hoffman, K. Bowenkamp, P. Huettl, J. Hudson, D. Martin, L.–F.H. Lin, G.A. Gerhardt, Glial cell line-derived neurotrophic factor reverses toxin-induced injury to

midbrain dopaminergic neurons in vivo, Neurosci. Lett. 182 (1994) 107-111.

[24] S. Jing, D. Wen, Y. Yu, P.L. Holst, Y. Luo, M. Fang, R. Tamir, L. Antonio, Z. Hu, R. Cupples, J.–C. Louis, S. Hu, B.W. Altrock, G.M. Fox, GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF, Cell 85 (1996) 1113-1124.

[25] S.-L. Lee, J.-K. Kim, D.-S. Kim, H.-J. Cho, Expression of mRNAs encoding full-length and truncated trkB receptors in rat dorsal root ganglia and spinal cord following peripheral inflammation, NeuroReport 10 (1999) 2847-2851.

[26] L.–F.H. Lin, D.H. Doherty, J.D. Lile, S. Bektesh, F. Collins, GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons, Science 260 (1993) 1130-1132.

[27] H.T.J. Mount, D.O. Dean, J. Alberch, C.F. Dreyfus, I.B.Black, Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells, Proc. Natl. Acad. Sci. USA 92 (1995) 9092-9096.

[28] C. Nozaki, N. Asai, H. Murakami, T. Iwashita, Y. Iwata, K. Horibe, R.D. Klein, A. Rosenthal, M. Takahashi, Calcium-dependent Ret activation by GDNF and neurturin, Oncogene 16 (1998) 293-299.

[29] R.W. Oppenheim, L.J. Houenou, J.E. Johnson, L.–F.H. Lin, L. Li, A.C. Lo, A.L.

Newsome, D.M. Prevette, S. Wang, Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF, Nature 373 (1995) 344-346.

[30] G. Pages, P. Lenormand, G. L'Allemain, J.–C. Chambard, S. Meloche, J. Pouyssegur, Mitogen-activated protein kinases p42mapk and p44mapk are required for fibloblast proliferation, Proc. Natl. Acad. Sci. USA 90 (1993) 8319-8323.

[31] S. Pahlman, A.–I. Ruusala, L. Abrahamsson, M.E.K. Mattsson, T. Esscher, Retinoic acid-induced differentiation of cultured human neuroblastoma cells: A comparison with phorbolester-induced differentiation, Cell Diff. 14 (1984) 135-144.

[32] K. Pong, R.Y. Xu, W.F. Baron, J.–C. Louis, K.D. Beck, Inhibition of phoshatidylinositol 3-kinase activity blocks cellular differentiation mediated by glial cell line-derived neurotrophic factor in dopaminergic neurons, J. Neurochem. 71 (1998) 1912-1919.

[33] D. Poteryaev, A. Titievsky, Y.F. Sun, J. Thomas-Crusells, M. Lindahl, M. Billaud, U. Arumae, M. Saarma, GDFN triggers a novel Ret-independent Src kinase family-coupled signaling via a GPI-linked GDNF receptor α1, FEBS Lett. 463 (1999) 63-66.

[34] C. Segouffin-Cariou, M. Billaud, Transforming ability of MEN2A-RET requires activation of the phosphatidylinositol 3-kinase/AKT signaling pathway, J. Biol. Chem. 275 (2000) 3568-3576.

[35] R.A. Segal, M.E. Greenberg, Intracellular signaling pathways activated by neurotrophic

factors, Annu. Rev. Neurosci. 19 (1996) 463-489.

[36] R.M. Soler, X. Dolcet, M. Encinas, J. Egea, J.R. Bayascas, J.X. Comella, Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons, J. Neurosci. 19 (1999) 9160-9169.

[37] A. Tomac, E. Lindqvist, L.–F.H. Lin, S.O. Ogren, D. Young, B.J. Hoffer, L. Olson, Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo, Nature 373 (1995) 335-339.

[38] J. J. S. Treanor, L. Goodman, F. de Sauvage, D.M. Stone, K.T. Poulsen, C.D. Beck, C. Gray, M.P. Armanini, R.A. Pollock, F. Hefti, H.S. Phillips, A. Goddard, M.W. Moore, A. Buj-Bello, A.M. Davies, N. Asai, M. Takahashi, R. Vandlen, C.E. Henderson, A. Rosenthal, Characterization of a multicomponent receptor for GDNF, Nature 382 (1996) 80-83.

[39] R. Treisman, Regulation of transcription by MAP kinase cascades, Curr. Opin. Cell Biol. 8 (1996) 205-215.

[40] M. Trupp, C. Raynoschek, N. Belluardo, C.F. Ibanez, Multiple GPI-anchored receptors control GDNF-dependent and independent activation of the c-Ret receptor tyrosine kinase, Mol. Cell. Neurosci. 11 (1998) 47-63.

[41] M. Trupp, R. Scott, S.R. Whittemore, C.F. Ibanez, Ret-dependent and independent mechanisms of glial cell line-derived neurotrophic factor signaling in neuronal cells, J. Biol. Chem. 274 (1999) 20885-20894.

[42] S. Volarevic, G. Thomas, Role of S6 phosphorylation and S6 kinase in cell growth, Prog. Nucleic Acid Res. Mol. Biol. 65 (2001) 101-127.

[43] D.H. van Weering, J.L. Bos, Glial cell line-derived neurotrophic factor induces Ret-mediated lamellipodia formation, J. Biol. Chem. 27 (1997) 249-254.

[44] C.A. Worby, Q.C. Vega, Y. Zhao, H.H.–J. Chao, A.F. Seasholtz, J.E. Dixon, Glial cell line-derived neurotrophic factor signals through the Ret receptor and activate mitogen-activated protein kinase, J. Biol. Chem. 271 (1996) 23619-23622.

[45] D.S. Worley, J.M. Pisano, E.D. Choi, L. Walus, C.A. Hession, R.L. Cate, M. Sanicola, S.J. Birren, Developmental regulation of GDNF response and receptor expression in the enteric nervous system, Development 127 (2000) 4383-4393.

[46] J. Xing, D.D. Ginty, M.E. Greenberg, Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase, Science 273 (1996) 959-963.

[47] Q. Yan, C. Matheson, O.T. Lopez, In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons, Nature 373 (1995) 341-344.

Fig. 1. GDNF stimulates the proliferation of SH-SY5Y cells. The cells (5000 cells/well) were cultured in 96-well plates for 2 days in 100 µl of medium/well. After the treatment of cells with various concentrations of GDNF, the WST-1 assay was performed with a kit according to the manufacturer's protocol (Wako Pure Chemical Industries). Each value is the mean ± SD of eight experiments. *$p < 0.01$ for difference from control.

Fig. 2. GDNF has similar mitogenic effects on SH-SY5Y cells in the absence or presence of retinoic acid (RA), a compound that strongly induces Ret protein levels. (A) WST-1 assay for cell proliferation. SH-SY5Y cells (5000 cells/well) were cultured in 96-well plates for 3 days in 100 µl of medium/well. The cells were treated with GDNF (100 ng/ml), RA (1 µM) or GDNF plus RA for the indicated periods, and cell proliferation was assessed with the WST-1 assay according to the manufacturer's protocol (Wako Pure Chemical Industries). (B) Representative phase-contrast photomicrographs of cultures bathed in basal medium (control), GDNF, RA, or GDNF plus RA. SH-SY5Y cells were maintained in culture in the presence of GDNF (50 ng/ml), RA (1 µM) or GDNF plus RA for 4 days. (C) RA strongly induces both short and long form of Ret protein, but not GFRα1 protein. SH-SY5Y cells were exposed to 1 µM RA for 0, 1, 2, or 5 days or were cultured for 1 day with increasing concentrations of RA. Following treatment, the cells were lysed and equal amounts (40 µg) of protein were

separated via SDS-PAGE and analyzed using Western blotting. Anti-monoclonal GDNFRα antibody (Transduction Laboratory) was used for the immunodetection of GFRα1. (D) RA-induced Ret protein responds to GDNF. SH-SY5Y cells cultured in the absence or presence of 1.5 μM RA for 1 day and stimulated with 50 ng/ml GDNF for 10 min. Ret was immunoprecipitated with Ret antibodies and analyzed using Western blotting and Ret and phosphotyrosine antibodies.

Fig. 3. GDNF has no effect on the proliferation of SK-N-MC, Neuro2a and PC12 cells. SK-N-MC cells (2000 cells/well) and Neuro2a cells (1000 cells/well) were cultured in 96-well plates for 1 day in 100 μl of medium/well, and treated with GDNF (10, 50, or 100 ng/ml) for the indicated periods. PC12 cells (3000 cells/well) were cultured in 96-well plates for 2 days in 100 μl of medium/well, and treated with GDNF (100 ng/ml) for the indicated periods. Cell proliferation was assessed with the WST-1 assay as described above.

Fig. 4. Both Ret and GFRα1 are necessary to stimulate (A) the Akt/p70S6 and (B) ERK pathways. SH-SY5Y(RA) cells were pretreated with 1.5 μM of RA for 24 h. The cells were stimulated with GDNF (100 ng/ml) for 10 min. Cells were lysed and equal amounts (30 μg)

of protein were separated on SDS-PAGE and analyzed using Western blotting. The expression of Ret and GFRα1 in various cell lines was determined using (C) Western blotting and (D) RT-PCR .

Fig. 5. (A) Comparison of p70S6K and ERK pathway activation in untreated and RA-treated SH-SY5Y cells. SH-SY5Y cells were treated with RA (1.5 μM) for 1 day and were stimulated with GDNF (100 ng/ml) for the indicated periods. Cells were lysed and equal amounts (40 μg) of protein were separated on SDS-PAGE and analyzed using Western blotting. (B) Induction of immediate-early gene products by GDNF in RA-treated SH-SY5Y cells. The cells were treated with RA (1.5 μM) for 1 day and were stimulated with GDNF (50 ng/ml) for the indicated periods. Immediate-early gene products were assessed using SDS-PAGE and Western blotting. (C) Activation of Rsk2 and CREB by GDNF in RA-treated SH-SY5Y cells. The cells were treated with RA (1.5 μM) for 1 day and were stimulated with GDNF (100 ng/ml) for the indicated periods. Rsk2 and CREB phosphorylation was assessed using SDS-PAGE and Western blotting.

Fig. 6. (A) Rapamycin completely blocks both GDNF-induced and RA-, GDNF-induced cell proliferation. SH-SY5Y cells (5000 cells/well) were cultured in 96-well plates for 3 days in

100 µl of medium/well. The cells were treated with GDNF (100 ng/ml) and/or rapamycin (100 nM) in the absence or presence of RA (1.5 µM) for the indicated periods, and cell proliferation was assessed with the WST-1 assay as described above. (B) PD98059 does not bolck GDNF-induced cell proliferation. SH-SY5Y cells (5000 cells/well) were cultured in 96-well plates for 2 days in 100 µl of medium/well. The cells were treated with GDNF (100 ng/ml) and/or PD98059 (20 µM) for the indicated periods, and cell proliferation was assessed with the WST-1 assay as described above. (C) Effect of rapamycin (RM) on GDNF-induced activation of Erk, Akt and p70 S6 kinase. SH-SY5Y cells, treated with RA (1.5 µM) for 24 h, were preincubated with rapamycin (100 nM) for 30 min and then stimulated with GDNF (100 ng/ml) for 30 min. Equal amounts (40 µg) of protein were separated on SDS-PAGE and analyzed by Western blotting.

Fig. 7 Schematic illustration of the intracellular signaling pathway in SH-SY5Y cells in the absence or presence of RA

Hirata & Kiuchi
Fig. 1

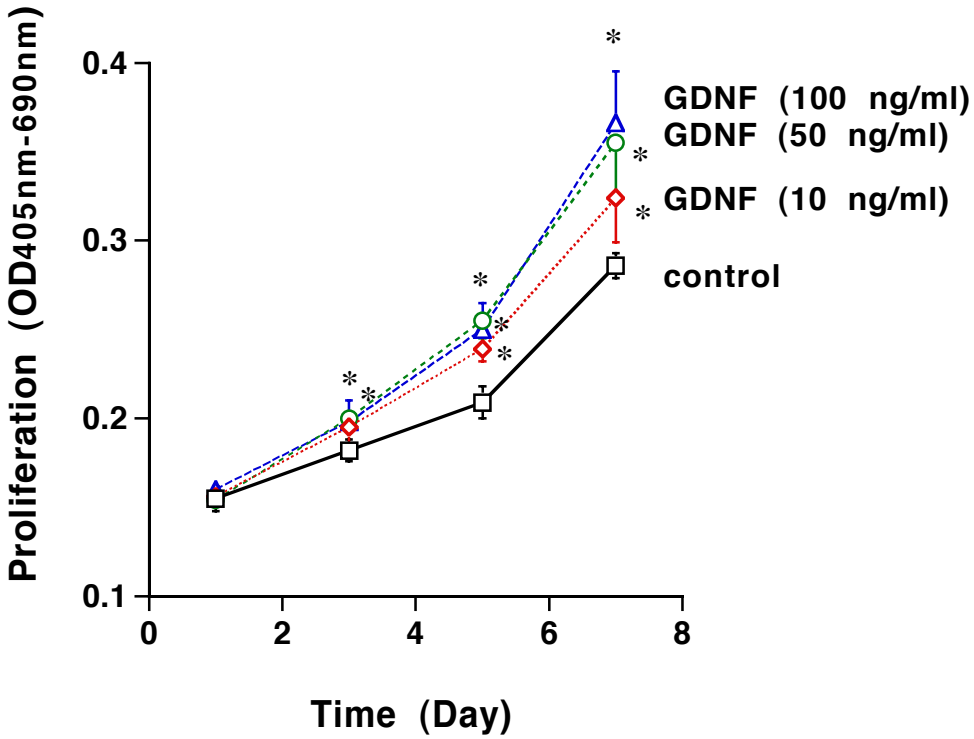
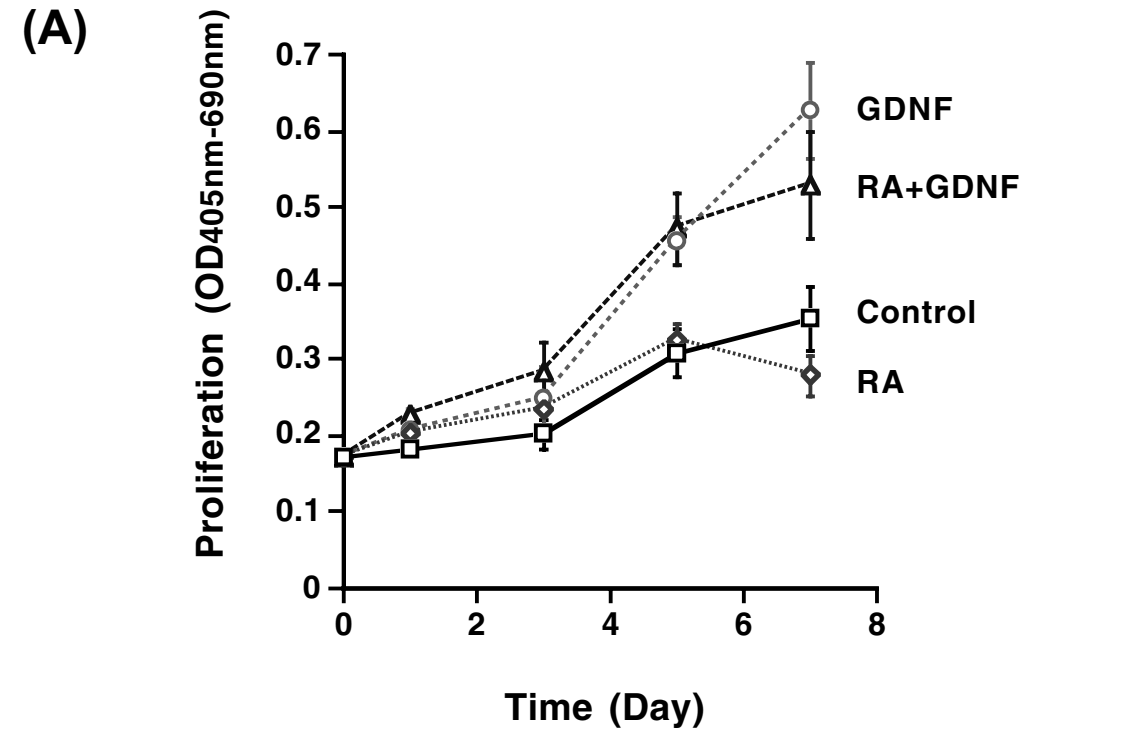


Fig. 2


(A)
Proliferation (OD405nm-690nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
Time (Day)
GDNF
RA+GDNF
Control
RA

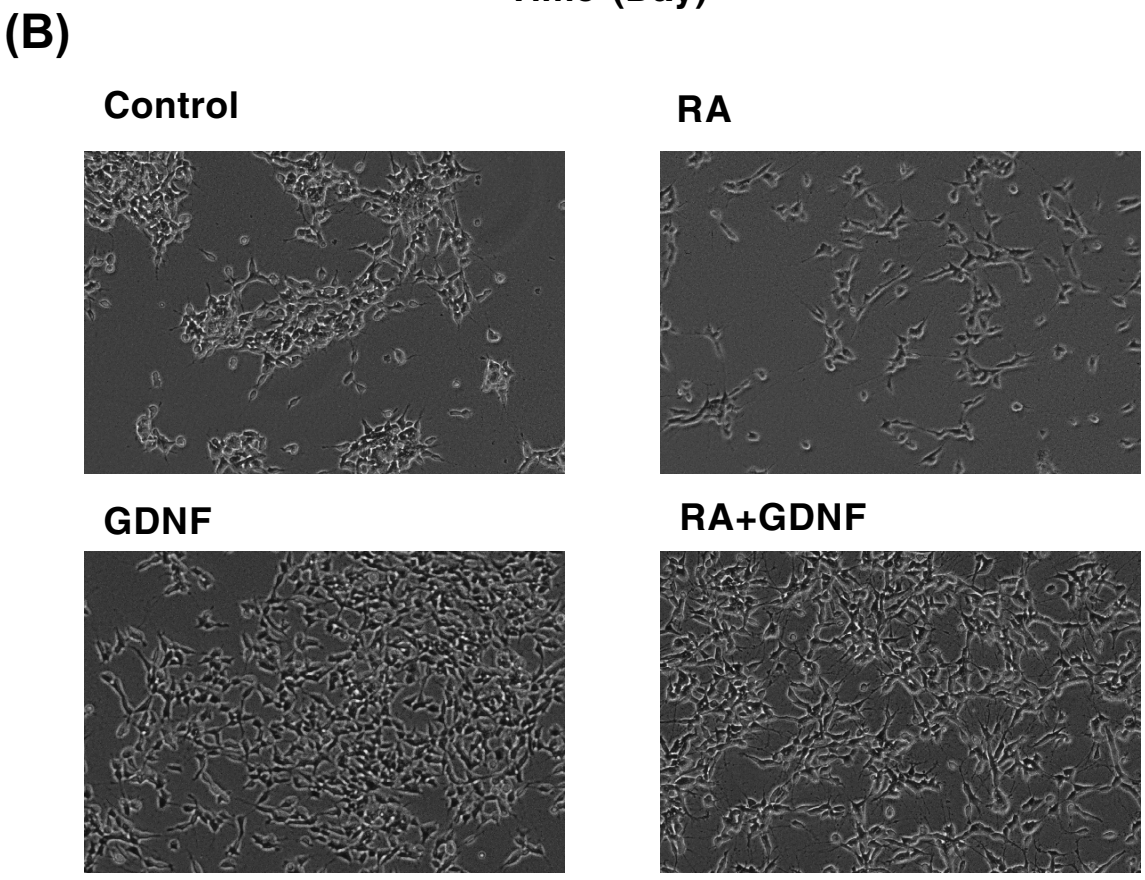
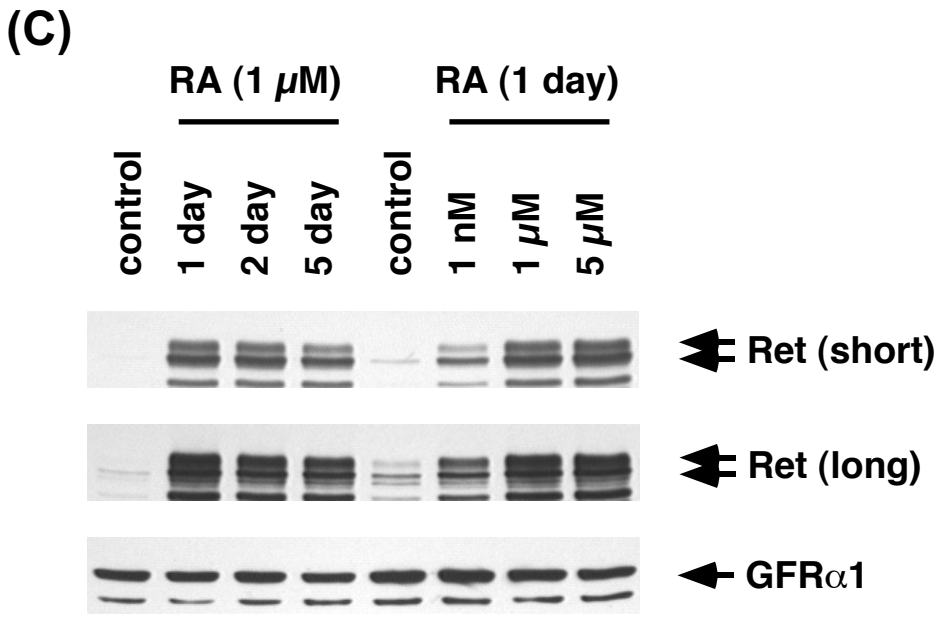

(B)
Control
RA
GDNF
RA+GDNF

Fig. 2


(C)
RA (1 μM)
RA (1 day)
control
1 day
2 day
5 day
control
1 nM
1 μM
5 μM
Ret (short)
Ret (long)
GFRα1

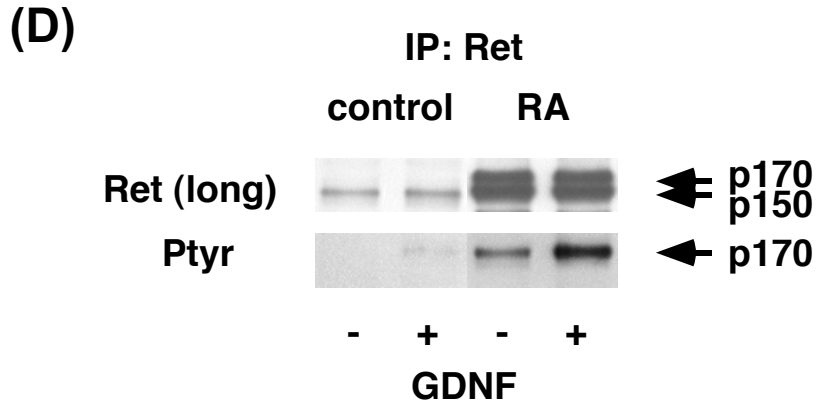

(D)
IP: Ret
control
RA
Ret (long)
Ptyr
p170
p150
p170
-
+
-
+
GDNF

Fig. 3

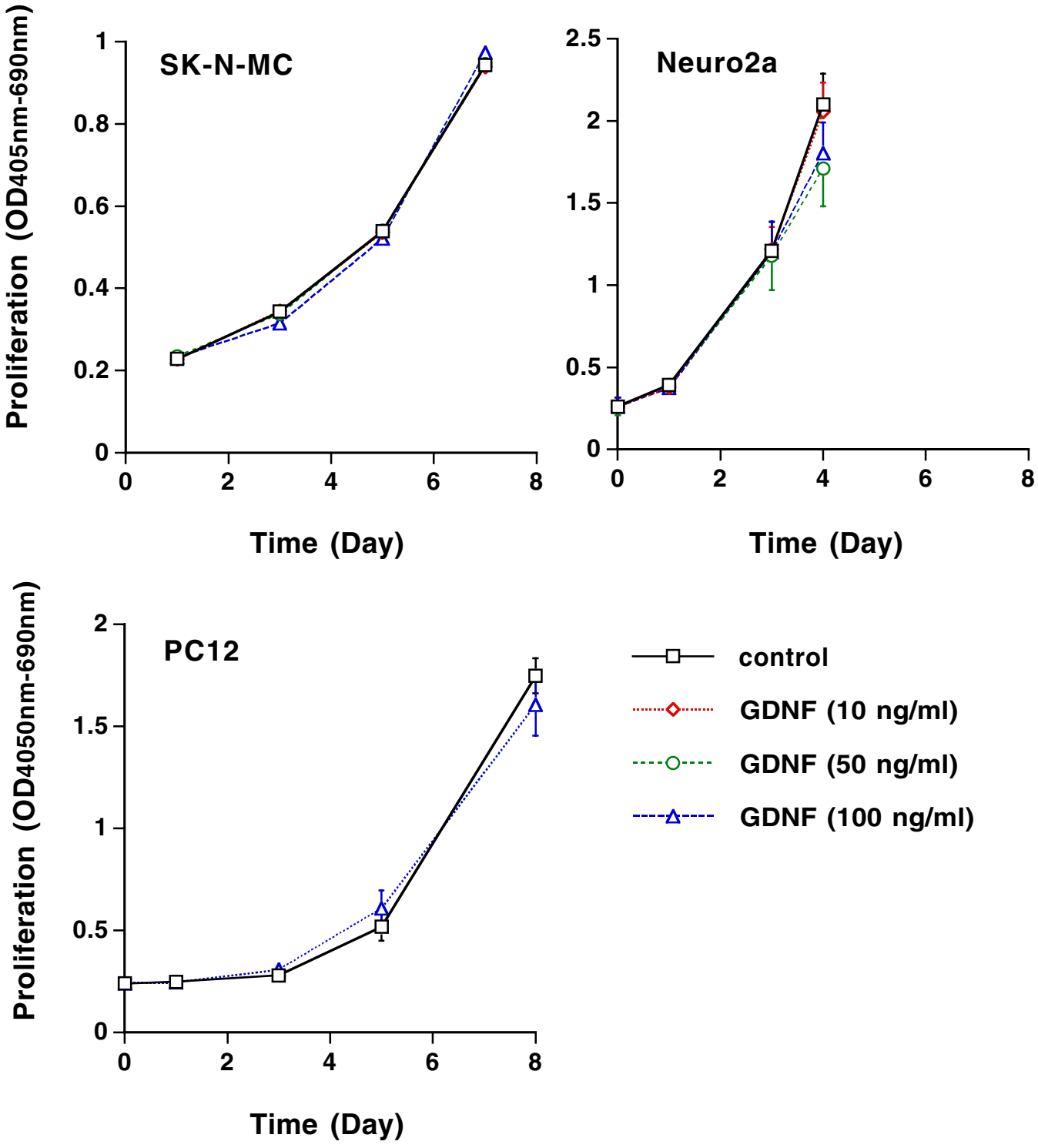

SK-N-MC
Neuro2a
PC12
Proliferation (OD405nm-690nm)
Proliferation (OD4050nm-690nm)
Time (Day)
Time (Day)
Time (Day)
control
GDNF (10 ng/ml)
GDNF (50 ng/ml)
GDNF (100 ng/ml)

Fig. 4

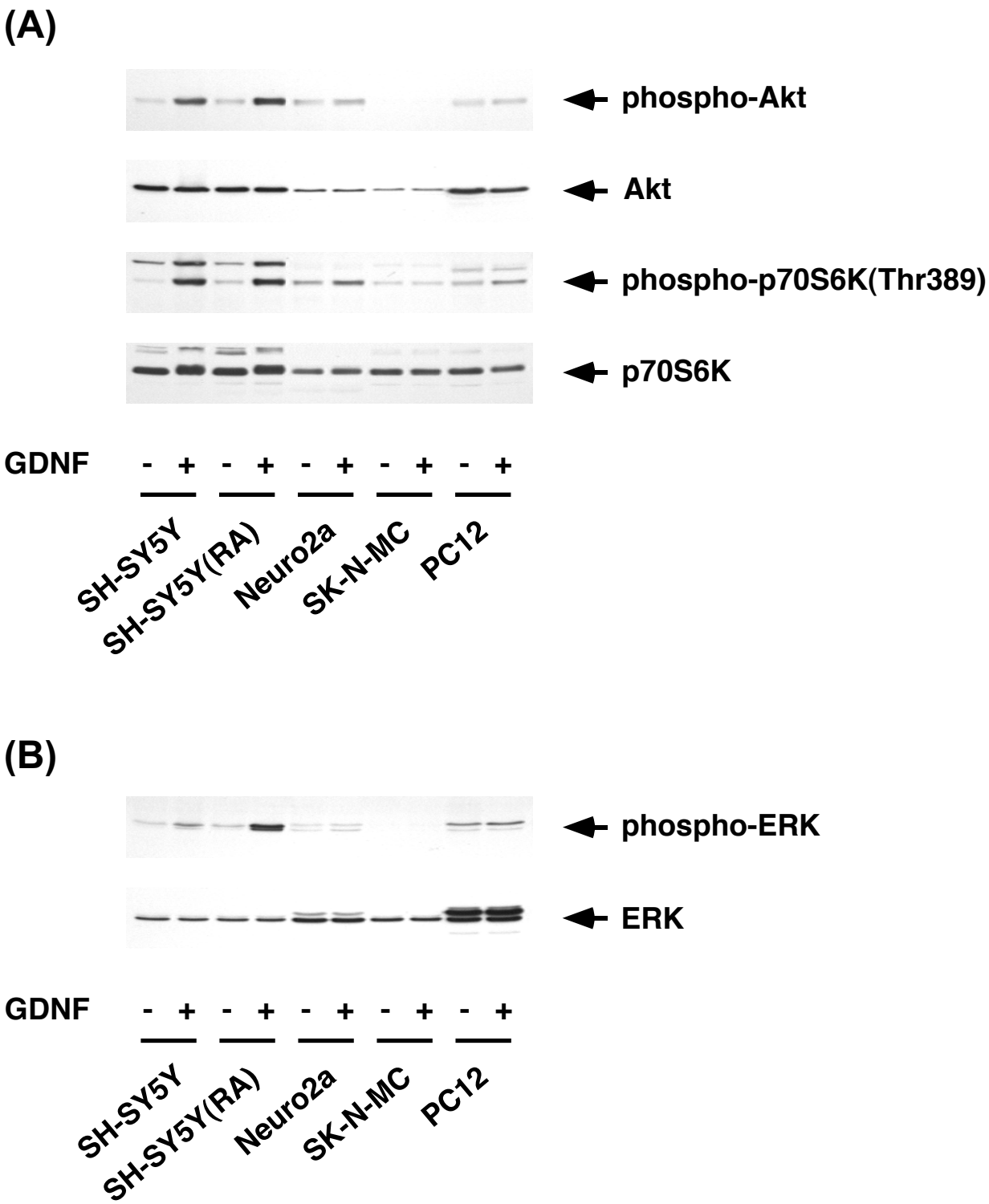
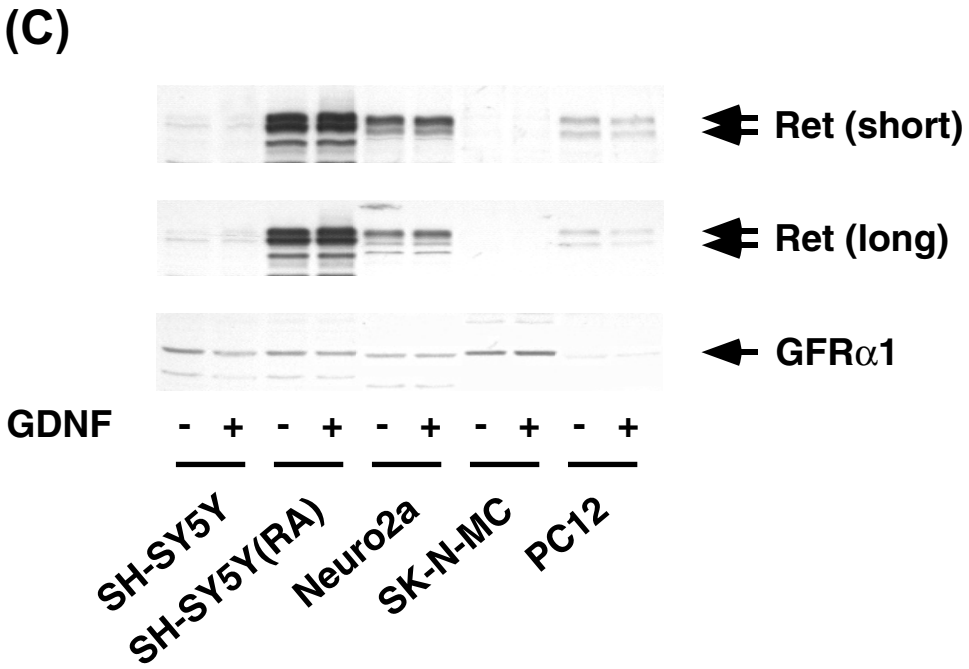

(A)
phospho-Akt
Akt
phospho-p70S6K(Thr389)
p70S6K
GDNF
- + - + - + - + - +
SH-SY5Y
SH-SY5Y(RA)
Neuro2a
SK-N-MC
PC12
(B)
phospho-ERK
ERK
GDNF
- + - + - + - + - +
SH-SY5Y
SH-SY5Y(RA)
Neuro2a
SK-N-MC
PC12

Fig. 4


(C)
Ret (short)
Ret (long)
GFRα1
GDNF
- + - + - + - + - +
SH-SY5Y
SH-SY5Y(RA)
Neuro2a
SK-N-MC
PC12

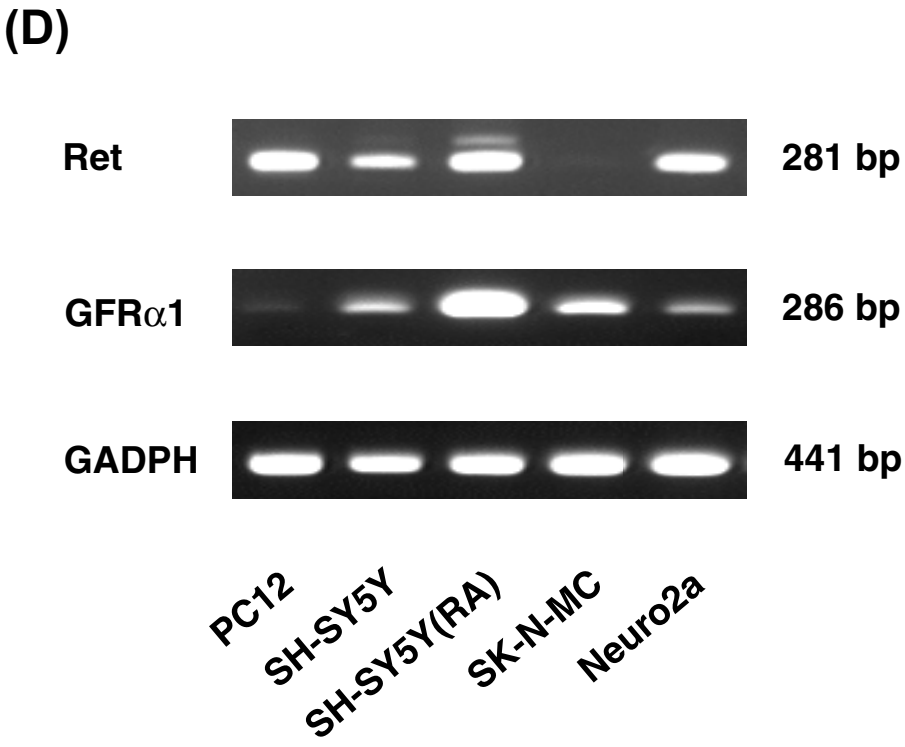
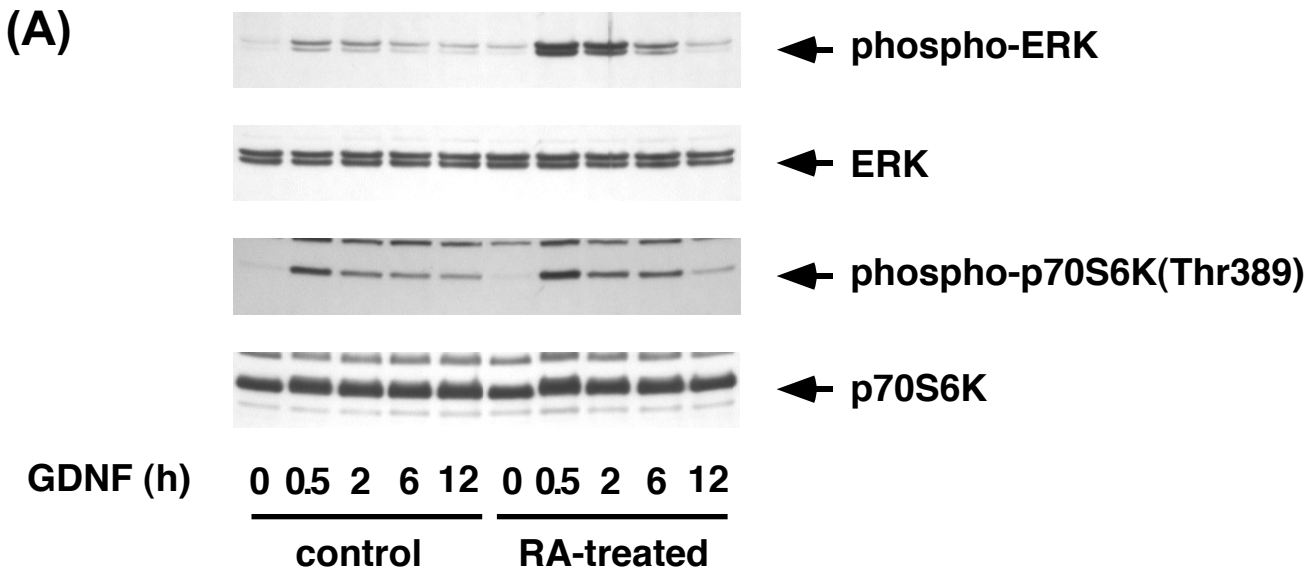

(D)
Ret
281 bp
GFRα1
286 bp
GADPH
441 bp
PC12
SH-SY5Y
SH-SY5Y(RA)
SK-N-MC
Neuro2a

Fig. 5


(A)
phospho-ERK
ERK
phospho-p70S6K(Thr389)
p70S6K
GDNF (h)
0 0.5 2 6 12 0 0.5 2 6 12
control
RA-treated

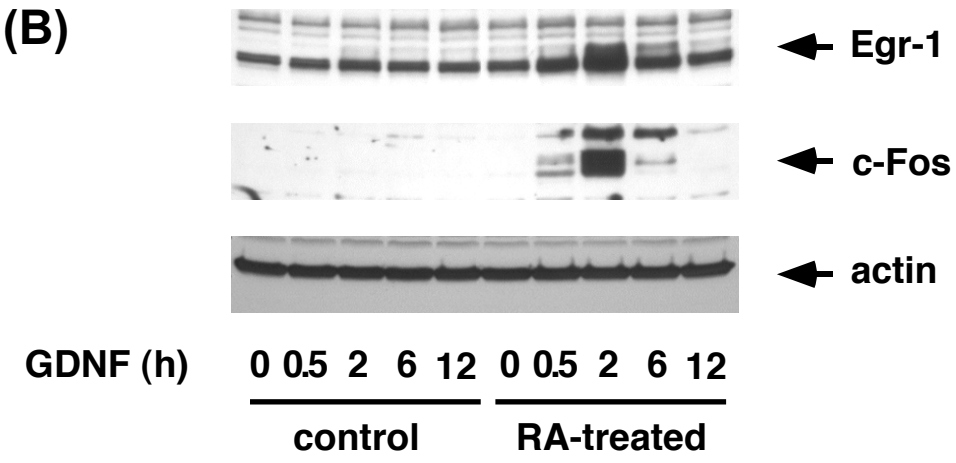

(B)
Egr-1
c-Fos
actin
GDNF (h)
0 0.5 2 6 12 0 0.5 2 6 12
control
RA-treated

Fig. 5

(C)

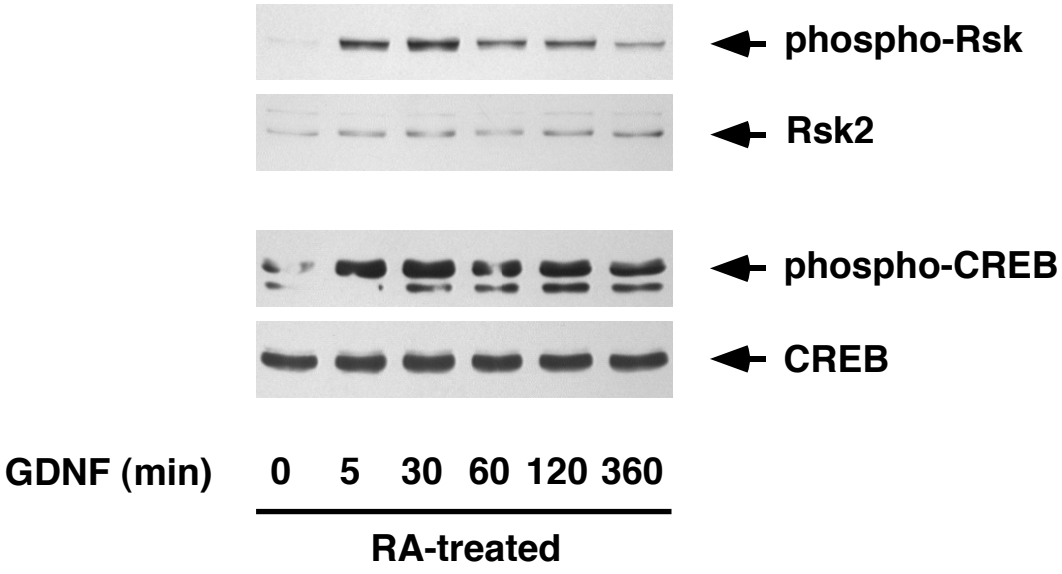
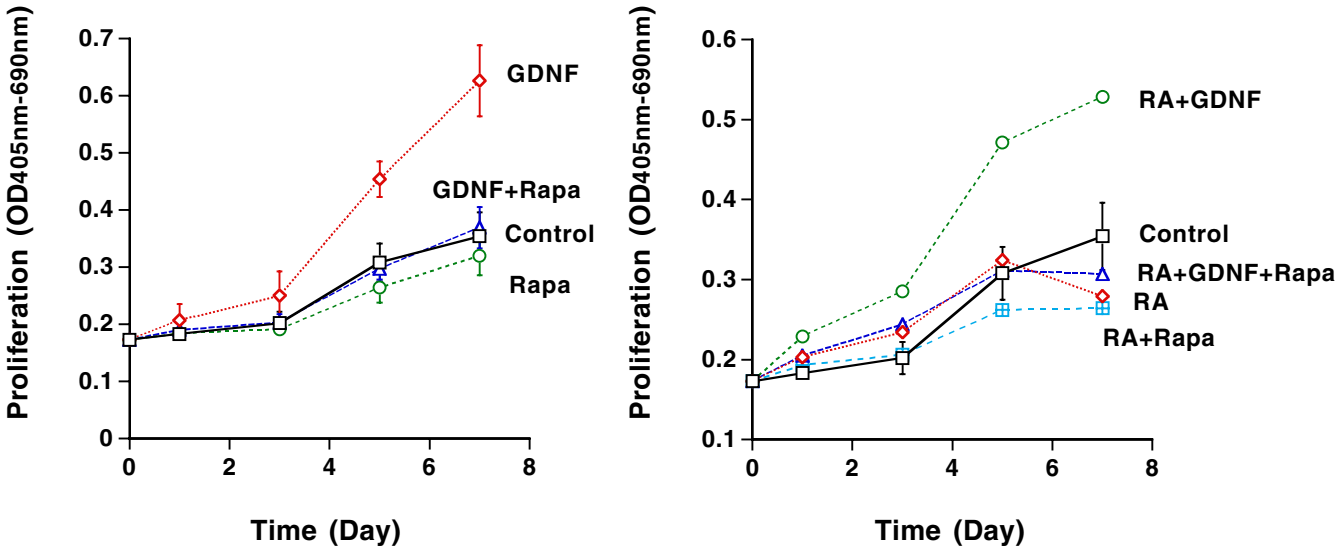

phospho-Rsk
Rsk2
phospho-CREB
CREB
GDNF (min)
0
5
30
60
120
360
RA-treated

Fig. 6

(A)


Proliferation (OD405nm-690nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
Time (Day)
GDNF
GDNF+Rapa
Control
Rapa
Proliferation (OD405nm-690nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
Time (Day)
RA+GDNF
Control
RA+GDNF+Rapa
RA
RA+Rapa

(B)

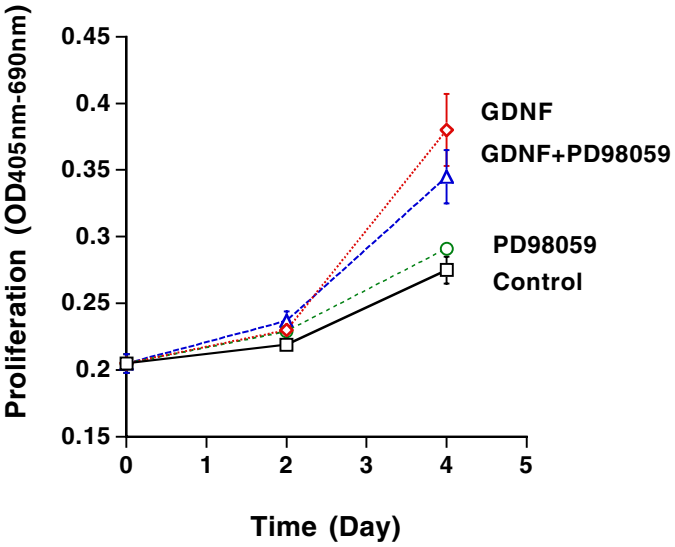

Proliferation (OD405nm-690nm)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0
1
2
3
4
5
Time (Day)
GDNF
GDNF+PD98059
PD98059
Control

Fig. 6

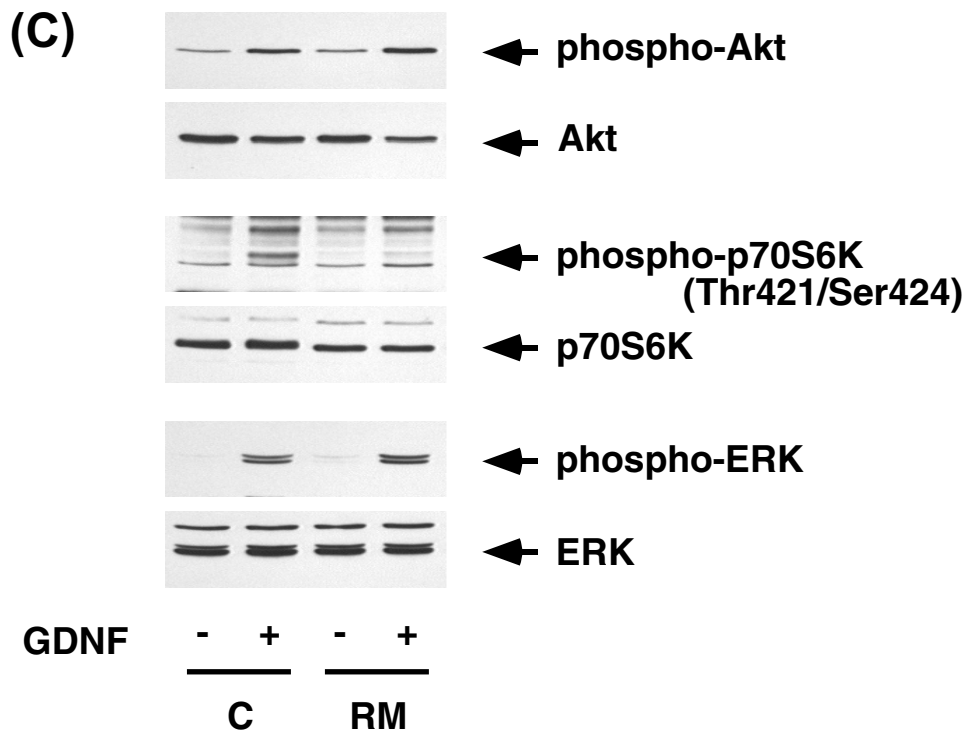
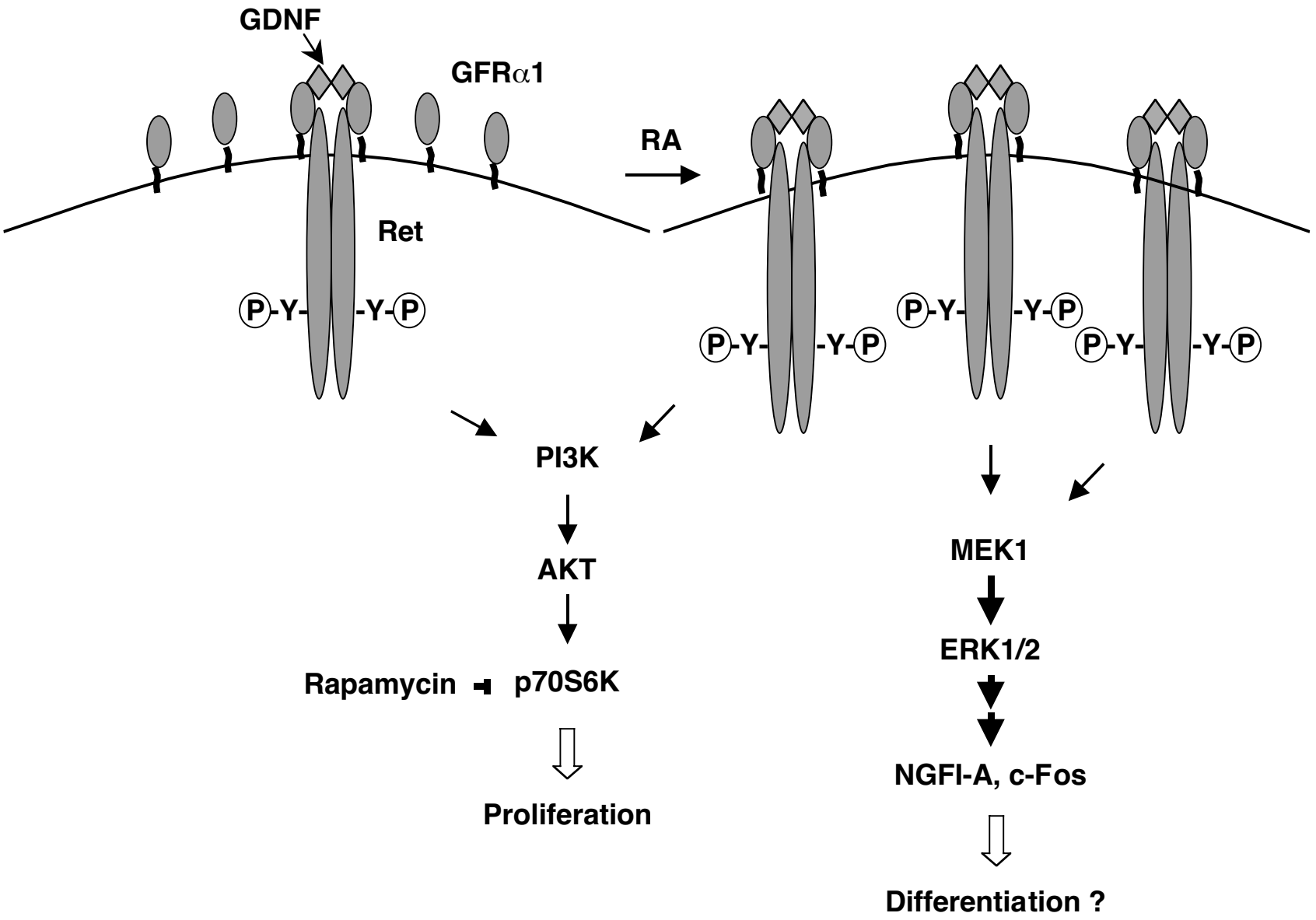

(C)
phospho-Akt
Akt
phospho-p70S6K
(Thr421/Ser424)
p70S6K
phospho-ERK
ERK
GDNF
-
+
-
+
C
RM

Fig. 7


GDNF
GFRα1
RA
Ret
P-Y-
-Y-P
PI3K
AKT
Rapamycin
p70S6K
Proliferation
MEK1
ERK1/2
NGFI-A, c-Fos
Differentiation ?