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Enhancement of tyrosine hydroxylase phosphorylation and activity by glial cell line-derived neurotrophic factor

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Running title: GDNF increases TH phosphorylation and enzymatic activity

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Summary

Although glial cell-line derived neurotrophic factor (GDNF) acts as a potent survival factor for dopaminergic neurons, it is not known if GDNF can directly alter dopamine synthesis. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for dopamine biosynthesis, and its

activity is regulated by phosphorylation on three seryl residues: Ser19, Ser31 and Ser40. Using a TH expressing human neuroblastoma cell line and rat primary mesencephalic neuron cultures, the present study examined if GDNF alters the phosphorylation of TH and if these changes are accompanied by increased enzymatic activity. Exposure to GDNF did not alter TH protein level in either neuroblastoma cells or in primary neurons. However, significant increases in the phosphorylation of Ser31 and Ser40 were detected within minutes of GDNF application in both cell types. Enhanced Ser31 and Ser40 phosphorylation was associated with increased TH activity, but not dopamine synthesis, in neuroblastoma cells possibly due to absence of L-aromatic amino acid decarboxylase activity in these cells. In contrast, increased phosphorylation of Ser31 and Ser40 was found to enhance dopamine synthesis in primary neurons. Pharmacological experiments show that Erk and PKA phosphorylate Ser31 and Ser40, respectively, and that their inhibition blocked both TH phosphorylation and activity. Our results indicate that, in addition to its role as a survival factor for dopaminergic neurons, GDNF can directly increase dopamine synthesis.

Key words: tyrosine hydroxylase, phosphorylation-specific antibodies, dopamine synthesis, catecholamine, extracellular signal-regulated kinase, and growth factor

Abbreviation: TH: tyrosine hydroxylase, GDNF: glial cell line-derived neurotrophic factor, Erk: extracellular signal-regulated kinase, PKA: protein kinase A, MEK: MAP/Erk kinase, RpcAMP: Rp-adenosine-3', 5'-cyclic mono-phosphorothioate , L-DOPA: 3,4-dihydroxy-L-phenyl alanine

Introduction:

Dopamine, a neurotransmitter of the mesostriatal, mesolimbic, and mesocortical neural projections, regulates various neurological functions including memory, attention, motivation, reward, and motor control. Alterations in the levels of this neurotransmitter have been linked to pathological conditions such as Parkinson's disease, schizophrenia, psychosis, drug dependence, dementia and attention deficit. Glial cell line-derived neurotrophic factor (GDNF) is a potent survival factor for dopamine neurons and is necessary for differentiation and maintenance of this phenotype. GDNF administration protects dopaminergic neurons from neurotoxin- and axotomy-induced death. These beneficial effects of GDNF have led to the suggestion that this trophic factor could be used as a therapeutic agent for the treatment of Parkinson's disease (1).

In addition to preventing the death of dopaminergic neurons, several studies have reported that GDNF can enhance dopamine levels and increase the quantal size of small synaptic vesicles in dopaminergic neurons (2, 3). One possible mechanism for the increases in dopamine levels and quantal size is stimulation of tyrosine hydroxylase [EC 1.14.16.2; tyrosine 3-monooxygenase; L-tyrosine tetrahydropteridine: oxidoreductase (3-hydroxylating); TH] activity. TH is the rate-limiting enzyme in the biosynthesis of dopamine, and therefore, the activity of this enzyme is likely to be a key determinant of dopamine levels. Although there have been reports of higher TH levels and more TH positive neurons in physically or chemically lesioned animals treated with GDNF, others indicate that GDNF cannot reverse injury-induced decreases in TH levels (4, 5, 6). These apparently contradictory results are likely to be caused by two factors: 1) difficulty in distinguish the effect of GDNF as a survival factor from its ability to modulate dopamine synthesis in these *in vivo* studies; and 2) TH protein levels may not directly reflect its activity and dopamine biosynthesis.

While enhanced transcription and translation can increase TH protein levels, the enzymatic activity is regulated by phosphorylation of the protein (7). Phosphorylation of seryl residues (Ser19, Ser31 and Ser40) has been observed both in vitro and in situ, and protein kinases that phosphorylate each of these sites have been identified in part (8). These studies report that phosphorylation at Ser31 and Ser40 correlates with stimulation of dopamine synthesis (9). However, the effect of GDNF on phosphorylation of TH and its enzymatic activity has not been examined. The present study uses a TH expressing human neuroblastoma cell line and rat primary mesencephalic neuronal cultures in order to examine the effect of GDNF on TH phosphorylation. TH enzymatic activity and dopamine synthesis were measured to examine if GDNF-mediated alterations in TH phosphorylation are accompanied by changes in enzymatic activity and dopamine synthesis.

Experimental procedures:

Materials. A BE(2)-C human neuroblastoma cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). Human recombinant GDNF and rat recombinant GDNF were purchased from R&D systems (Minneapolis, MN). Anti-synaptophysin polyclonal antibody and Alexa dye-conjugated secondary antibody were purchased from DakoCytomation (Carpinteria, CA) and Molecular Probes (Eugene, OR), respectively. Pan-specific anti-TH polyclonal antibody, phospho-specific antibody for rat TH (Ser31 and Ser40), and Anti-NeuN monoclonal antibody were from Chemicon (Temecula, CA). Anti-phospho-Erk1/2 polyclonal antibody and LY294002 were from Cell Signaling Technology (Beverly, MA). Okadaic acid and PD098059 were from Calbiochem (San Diego, CA). 3,5-[^{3}H]-L-tyrosine and [1-^{14}C]-L-tyrosine were obtained from Amersham Pharmacia Biotech (Buckinghamshire, England) and Moravek

Biochemicals (Brea, CA), respectively. Rp-cAMPS was from Biomol Research Laboratories (Plymouth Meeting, PA). U0126, D,L-6-Met-5,6,7,8-tetrahydropterine, L-tyrosine, ascorbic acid, and catalase were purchased from Sigma (Saint Louis, MO). Culture media and fetal bovine serum were from Life Technology (Rockville, MA).

BE(2)-C Cell Culture. Cells in culture were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), non-essential amino acids (NEAA), and an antibiotics/antimycotics mixture (100 units/mL penicillin G, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B) in Falcon culture flasks (Becton Dickinson, Franklin Lakes, NJ). It has been reported that high serum concentration induces dopamine phenotype in these cells (10). After four to six passages in 10% serum, the serum concentration was raised to 20% for additional two to four passages. Cells were then re-plated either in 35 mm Falcon culture dishes or in 24-well culture plates (Becton Dickinson) at a density of 1×10^5 / cm^2 and treated with 10 µM all-trans retinoic acid (RA) plus 20 % FBS for 6 days. Culture medium and RA were renewed every other day. Exposure to retinoic acid for six days resulted in differentiation of cells to a dopamine neuronal phenotype as determined by morphology and immunoreactivity for synaptophysin and TH. Differentiated cells were used for the TH phosphorylation and activity experiments.

Serum deprivation and GDNF treatment for BE(2)-C cells. Human recombinant GDNF was reconstituted in 0.1 % bovine serum albumin (BSA) in PBS (Life Technology) at a concentration of 50 µg/mL as recommended by the manufacturer and stored at -80 ^oC until use. Cells were washed with DMEM/F12 culture medium two times followed by incubation in DMEM/F12

supplemented with NEAA and antibiotics/antimycotics mixture with or without 50 ng/mL GDNF. Control cells received an equivalent amount of BSA. GDNF was maintained throughout the incubation period. Cells were harvested either immediately, 10 min, 30 min, 60 min, 3 hr, or 6 hr following treatment and lysed in a buffer containing 10 mM Tris pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.5 μM DTT, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM PMSF, and 0.1 μM okadaic acid. Extracts were snap-frozen and stored at -80 ^{o}C until needed. For the inhibition studies using the MAP kinase kinase (MEK) inhibitor U0126 (5 μM) or PD098059 (25 μM), or the phosphatidylinositol-3-kinase (PI3-kinase) inhibitor LY294002 (20 μM), cells were pre-incubated in the culture medium containing an inhibitor at the indicated concentration for an hour before serum deprivation. Following the incubation, cells were washed with DMEM/F12 followed by incubation in serum-deprived culture medium including the inhibitor in the presence or absence of GDNF for 60 min. Cells were lysed and extracts were prepared as described above. Prior to use, the cell lysate was sonicated and the amount of protein in each sample was measured by a Bradford assay using BSA as the standard.

Primary mesencephalic neuronal culture. Primary neurons were cultured from E15 Sprague-Dawley rat embryos. The ventral mesencephalic tissue was dissected out in an ice-cold DMEM/F12 culture medium followed by treatment with PBS (phosphate buffered-saline pH 7.4) containing 0.05 % trypsin and 0.53 mM EDTA (Gibco BRL) at 37 ^{o}C for 15 min. The treatment was terminated by adding an equal volume of the culture medium supplemented with 10 % fetal bovine serum. The tissue was resuspended in the culture medium and triturated using a 1 mL pipette tip 10 times followed by incubation on ice for 10 min. Suspended cells were removed and clumped materials at the bottom of the tube were again triturated using a 200 μL pipette tip

10 times and kept on ice for additional 10 min. Suspended cells were collected and combined, followed by centrifugation at 250 x g. Cells were cultured in DMEM/F12 supplemented with 10 % fetal bovine serum in poly-L-lysine-coated 35 mm culture dish or 24-well plate (Falcon) at a density of 3 x 10^5 cells/cm^2 for 36 hours. The cells were treated with 5 μM cytosine 1-β-D-arabinofuranoside (AraC, Sigma) for 24 hours, switched to the serum-free culture medium containing Neurobasal medium supplemented with B27 component, 2 mM GlutaMax-1, and the antibiotics/antimycotics mixture (Gibco BRL). Cells were maintained in the same medium for additional 10 days prior to use. Rat GDNF was added to the culture medium at a concentration of 50 ng/mL and the cells were incubated for 30 min unless otherwise described. Addition of protein kinase A inhibitor Rp-cAMPS (Rp-adenosine-3', 5'-cyclic mono-phosphorothioate, 50 μM) or U0126 (5 or 50 μM) to cultured neurons, cell lysis, and sample preparations were carried out as described above for BE(2)-C cells.

Immunohistochemical staining. For the immunohistochemical staining, cells were washed briefly in PBS and fixed with 4 % paraformaldehyde in PBS for an hour at 4 ^oC. Following fixation, cells were washed in 10 mM Tris-buffered saline pH 7.4 (TBS), permeabilized with ice-cold methanol for 5 min followed by blocking with 5 % normal goat serum for an hour at room temperature. Cells were incubated with pan-specific anti-TH polyclonal antibody (1:5000 dilution), anti-synaptophysin polyclonal antibody (1:5000 dilution), or anti-NeuN monoclonal antibody (1:2000 dilution) in TBS at 4 ^oC. Following overnight incubations, cells were washed three times in TBS and incubated with an Alexa 488-conjugated anti-rabbit-IgG or Alexa568-conjugated anti-mouse-IgG as suggested by the vendor. Immunoreactivity was detected using a

Leica DMIRB microscope and images were adjusted for size and labeled using Adobe *Photoshop 6.0*.

Production of phosphorylation-specific antibodies and western blotting. The following phosphorylated peptides (corresponding to the bovine TH sequence) were synthesized and used for immunizing rabbits. Phospho-Ser31: QAEAIM^{p}SPRF (identical to human TH-1 isoform), Phospho-Ser40: GRRQ^{p}SLIQDAR (human TH-1 sequence: GRRQSLIEDAR). Antibodies were sequentially purified first using phosphopeptide affinity columns, followed by removal of any residual non-phosphorylation-specific antibodies using non-phospho-peptide columns. Non-phosphorylated-specific antibodies for Ser31 and Ser40 were produced by immunizing rabbits with the appropriate peptides, and then purified using the nonphosphopeptide affinity chromatography followed by adsorption against a phosphopeptide column to remove cross-reacting antibodies. The affinity-purified antibodies were characterized by western blotting. Samples were resolved in a SDS-PAGE and transferred to an Immobilon-P (Millipore, Bedford, MA) membrane using a semi-dry transfer apparatus (Millipore). Membranes were blocked overnight in TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.05 % Tween-20) plus 5% BSA, and incubated with a primary antibody (1: 1,000 – 2,500 dilution for phospho- and non-phospho-TH antibodies, 1:50,000 for pan-specific TH antibody, or 1:2,000 for phospho-Erk1/2 antibody) for 3 hr at room temperature. The migration of TH was confirmed using a pan-specific polyclonal antibody, which recognized both the phosphorylated and unphosphorylated forms of TH (Chemicon). In addition, commercially available phosphorylation-specific antibodies for Ser31 or Ser40 (Chemicon), which recognize rat TH were also used to corroborate immunoreactivity in rat neuronal culture. Following incubation with the primary antibody, membranes were washed

three times 20 min each in TBST. Immunoreactivity was assessed by an alkaline phosphatase-conjugated secondary antibody and a CDP-star chemiluminescent substrate (Cell Signaling Technology). The optical density of the immunoreactive bands was measured utilizing ImageQuant band-analysis software (Amersham Biosciences, Piscataway, NJ). Prior to reprobing, blots were stripped by two 10 min washes in 50 mM NaOH at room temperature. The membrane was then washed extensively with TBST and reblocked for an hour in 2 % BSA prior to immunodetection.

TH activity and dopamine synthesis assays. TH catalyzes the hydroxylation of tyrosine to generate 3,4-dihydroxy-L-phenylalanine (L-DOPA) and water using D,L-6-Met-5,6,7,8-tetrahydropterine (Pt-H_4) as a cofactor. TH activity was measured by quantifying tritiated water production from 3,5-[^{3}H]-L-tyrosine (water assay), as previously described by Levine et al. (11), with minor modification. 35 μL of cell lysates were added to an equal volume of assay mixtures to yield a final reaction mixture containing 150 mM Tris-Malate buffer (pH 6.4), 0.35 μCi 3,5-[^{3}H]-L-tyrosine, 50 μM L-tyrosine, 5 mM ascorbic acid, 3 mM Pt-H_4, and 1,500 units catalase. After an incubation of 10 min at 37 ^{o}C, the reaction was stopped by cooling the samples on ice, followed by addition of 700 μL of 7.5 % activated charcoal in 1 N HCl. The samples were then centrifuged and the aqueous phase was recovered and mixed with 4 mL Universol (ICN Pharmaceuticals, Costa Mesa, CA) liquid scintillation fluid. Radioactivity was counted in a liquid scintillation analyzer. Blank values were obtained from identically prepared samples that did not contain cell lysate. The assays were performed in duplicate.

An assay to measure dopamine synthesis, which monitors $^{14}CO_2$ production following the conversion of [1-^{14}C]-L-tyrosine to dopamine, was performed as previously described by

Salvatore et al. (CO_2 assay)(12). BE(2)-C cells were cultured in 24-well plates. Following treatment, cells in each well were equilibrated in 200 µL HEPES-buffered saline (HBS) pH 7.4 (15 mM HEPES, 150 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM EGTA, 0.5 mM ascorbic acid, 5.6 mM glucose, 1 mM $MgCl_2$, and 1.9 mM K_2HPO_4) for 30 min at 37 ^oC. Each well was fitted with a section of Tygon tubing to enable collection of $^{14}CO_2$ generated during the enzymatic reaction. Ten µL HBS containing 0.1 µCi [1-^{14}C]-L-tyrosine was added in each well and incubated for 10 min at 37 ^oC. At the end of the incubation, 200 µL 20% trichloroacetate was added to terminate the reaction, a rubber stopper fitted with a suspended plastic well containing Whatman-3 filter paper saturated with Soluen-350 (Packard, Meriden, CT) was placed into the tubing. After allowing absorption of the generated $^{14}CO_2$ for 2 hr, the filter paper was transferred to 4 mL *Universol* liquid scintillation fluid. Radioactivity was counted as described above. Blank values were obtained from identically prepared samples that did not contain cells. The assays were performed in duplicate.

Calculation of fractions of phosphorylated and non-phosphorylated TH.

The fractions of phosphorylated and non-phosphorylated TH at a single site can be calculated using the fold changes for phospho and non-phospho TH immunoreactivities. This can be achieved provided: 1) the total amount of TH remains constant over time and 2) increase/decrease in immunoreactivity is linearly related to the amount of corresponding antigen on the western blot. Let P_0 and Pt represent the fraction of phosphorylated TH at time zero, and at a later time t, respectively. N_0 and Nt represent the fractions of nonphosphorylated TH at time zero and at time t, respectively.

$$P_0 + N_0 = 1 \qquad \text{[eq 1]}$$

$$Pt + Nt = 1 \qquad \text{[eq 2]}$$

x (= Pt/P_0) represents the fold change in phosphorylated TH immunoreactivity at time t and y (= Nt/N_0) represents the fold change in nonphosphorylated TH immunoreactivity at the same time point.

$$P_0 + N_0 = Pt + Nt = xP_0 + yN_0 \qquad \text{[eq 3]}$$

$$N_0 - yN_0 = xP_0 - P_0$$

Therefore, $N_0 = (x - 1)P_0/(1 - y)$ [eq 4]

Equations 1 and 4 can be solved to obtain

$$P_0 = (1-y)/(x-y) \qquad \text{[eq 5]}$$

and

$$Pt = x\,(1-y)/(x-y) \qquad \text{[eq 6]}$$

Statistical analysis. Statistical significance was determined by a repeated-measures analysis of variance (ANOVA) followed by post-hoc analysis. Data were considered significant at $P<0.05$. Statistical analysis was performed using either the integrated optical densities (western blot) or scintillation counts (enzyme activity assay).

Results:

Retinoic acid (RA) treatment induces neuronal morphology and increases the expression of synaptophysin and TH in BE(2)-C human neuroblastoma cells.

RA exposure has been shown to cause differentiation of BE(2)-C cells (10), (13), resulting in dopamine-like neurons. BE(2)-C cells were treated with 10 μM all trans-retinoic acid, and morphological changes were examined using phase-contrast microscopy as well as by

immunohistochemical staining for TH and synaptophysin, a synaptic-vesicle protein (Fig. 1a). Cells not exposed to RA possess short neurites and show weak synaptophysin and TH immunoreactivity (Fig 1a). Differentiation by RA is associated with cessation of proliferation and extensive branching of the neuronal processes. By 6 days post-RA exposure, the expression of synaptophysin and TH are markedly increased (Fig 1a). We next examined if this increase in TH immunoreactivity is accompanied by enhanced TH activity and dopamine synthesis. TH activity using cell extracts and dopamine synthesis in intact cells were measured as described in the Material and Methods section. Figure 1b shows that RA treatment significantly increases TH activity as compared to the untreated cells. In contrast, measurement of dopamine synthesis by monitoring $^{14}CO_2$ production did not show any detectable synthesis in either untreated or RA-treated cells (data not shown). This could be due to the absence of L-aromatic amino acid decarboxylase activity, which catalyzes the conversion of L-DOPA to dopamine in these cells. It has been reported that other neuroblastoma cell lines also lack this enzyme and do not synthesize dopamine (14). Dopamine inhibits its synthesis by directly binding to TH and this binding of dopamine is blocked when TH is phosphorylated on Ser40. Thus a lack of dopamine synthesis in BE(2)-C cells minimizes the involvement of Ser40 phosphorylation in regulating TH activity. These cells are therefore useful in isolating the contribution of Ser31 phosphorylation to TH axtivity.

Characterization of TH antibodies.

Phosphorylation- and non-phosphorylation-specific antibodies were generated to examine phosphorylation changes in response to treatments. The specificity of the phospho-specific antibodies were evaluated using bacterially expressed bovine TH and by western blot analysis.

Bacterially expressed proteins are not phosphorylated as bacteria lack protein kinases required to phosphorylate TH (15). Figure 1c shows that the pan-specific TH antibody detects bacterially expressed TH. As anticipated, the phosphorylation-specific antibodies for Ser40 or Ser31 did not detect bacterially expressed TH. In contrast, the non-phosphorylation-specific antibodies for Ser40 and Ser31 reacted with the TH protein. When BE(2)-C cell extracts were used, both phospho-specific antibodies detected a band slightly larger than the band detected in bovine adrenal gland protein extracts (Fig. 1d). This observation is consistent with the reported size difference between human and bovine TH (16, 17). The identity of this band was confirmed by reprobing the membranes with a pan-specific TH antibody (Fig. 1d, right). Neither the pan-specific, nor the phospho-specific, antibodies cross reacted with extracts from Jurkat T-cells, which do not express TH. Furthermore, preincubation of each of the phospho-specific antibodies with 20-fold molar excess of the phospho-peptide used for immunization blocked the corresponding immunoreactivity (Fig. 1d, lane 4). The linear range for TH immunoreactivity in western blots was determined for all antibodies using increasing amounts of protein samples. The immunoreactivity was found to increase in a linear manner with amounts of total protein ranging from 5 μg to 62 μg (data not shown). Subsequent experiments were performed using 10 to 30 μg total proteins.

GDNF treatment does not alter TH levels.

Although previous studies have shown that GDNF is a survival factor for dopamine neurons (4), (18), it is not clear if GDNF alters TH protein levels. GDNF binds to two receptors (GFRα1 and GFRα2) that recruit the Ret tyrosine kinase to the lipid raft (19). This results in the phosphorylation of Ret at multiple tyrosine residues that serve as docking sites for intracellular

signaling molecules (20, 21). Using mRNA prepared from both undifferentiated and differentiated cells, we tested if these cells express GDNF receptors by PCR. The mRNA for *ret* and *gfrα2*, but not for *gfrα1*, are present in both differentiated and undifferentiated BE(2)-C cells (data not shown).

To examine if exposure to GDNF changes TH protein levels, BE(2)-C cell extracts were prepared at different time points following serum deprivation and GDNF exposure, and analyzed by western blotting using the pan-specific TH antibody. The optical density of the immunoreactive band at each time point was normalized with respect to the zero time point and expressed as fold-change. A representative western blot and the summary data compiled from three independent experiments are shown in Figure 2a. The figure shows that pan-TH immunoreactivity does not significantly change as a result of serum deprivation or GDNF treatment at any of the time points examined.

GDNF treatment increases Ser31 and Ser40 phosphorylation.

The protein samples used to examine pan-TH immunoreactivity in Figure 2a were analyzed for changes in TH phosphorylation. Figure 2b shows representative western blots and summary data indicating that serum deprivation did not alter phospho-Ser40 immunoreactivity at any of the time points examined. In contrast, GDNF treatment significantly increased the phosphorylation of Ser40 as early as 30 min compared to both the serum deprived group (∗) and the zero time point samples (+) used as controls. This increase in phospho-Ser40 immunoreactivity was detected for up to 3 hr post-GDNF application (Fig. 2b). Changes in Ser40 phosphorylation were further examined by re-probing the membranes with Ser40 non-phosphorylation-specific antibodies. The immunoreactivity of non-phospho-Ser40 did not

change as a result of serum deprivation, but was significantly decreased as a result of GDNF treatment for up to 60 min (Fig. 2c). Consistent with the observed increases in immunoreactivity using the phospho-Ser40 antibody, the immunoreactivity using the non-phospho-Ser40 shows a corresponding decrease at the time points examined.

Figure 2d shows that serum deprivation increased phospho-Ser31 immunoreactivity at the 30 and 60 min time points. GDNF treatment significantly augmented phospho-Ser31 immunoreactivity with changes detected as early as 10 min post GDNF application and lasting for up to 3 hr. A corresponding decrease in non-phospho-Ser31 immunoreactivity was detected as a result of GDNF exposure, beginning as early as 10 min post-treatment and returning to control values by 3 hr (Fig. 2e).

Erk activity is required for Ser31, but not Ser40, phosphorylation.

Previous studies have shown that phosphorylation of TH on Ser31 enhances enzymatic activity by increasing the Vmax of the enzyme. In contrast, phosphorylation of Ser40 increases TH activity by increasing the rate of dissociation of inhibitory catecholamines from the enzyme (12), (22). Furthermore, in the absence of catecholamines, Ser40 phosphorylation does not increase TH activity (23). It has been reported that Erk can phosphorylate Ser31 (12), (22). Therefore, we examined if the increases in Ser31 phosphorylation are due to GDNF-mediated Erk activation. A representative western blot, and the summary data compiled from three independent experiments, showing the temporal change in phospho-Erk immunoreactivity are shown in Figure 3a. The phosphorylation of Erk reaches a maximum within 10 min and returns to control values by 6 hr following both serum deprivation and/or GDNF exposure. The

temporal profile of Erk activation is consistent with it being the Ser31 phosphorylating kinase. To examine if Ser31 and Erk phosphorylation co-vary at different concentrations of GDNF, a dose-response study was carried out. Phospho-Ser31 and phospho-Erk immunoreactivities showed parallel increases (subtracting changes caused by serum deprivation) in a dose-dependent manner up to 100 ng/mL GDNF (Fig. 3b).

To evaluate if a causal link exists between Erk activity and Ser31 phosphorylation, the MEK inhibitors U0126 and PD098059 were used. Figure 3c shows that both U0126 and PD098059 blocked the serum-deprived and GDNF-induced increases in phospho-Erk immunoreactivity. This was accompanied by a corresponding decrease in phospho-Ser31 immunoreactivity (Fig. 3c). Re-probing of these membranes with the pan-specific TH antibody indicated that the decrease in phospho-Ser31 immunoreactivity was not due to an overall change in the protein levels. To determine if PD098059 decrease Ser40 phosphorylation, membranes were re-probed with antibodies for phospho-Ser40. PD098059 did not alter the immunoreactivity of phospho-Ser40 (Fig 3c). In addition to stimulation of the Erk cascade, the binding of GDNF to its receptors activates phosphoinositides-3,4,5 (PI3)-kinase (24, 25, 26, 27). To examine if the PI3-kinase cascade contributes to Ser31 phosphorylation, LY294002 was utilized. Figure 3d shows that LY294002 did not alter phospho-Erk or phospho-Ser31 immunoreactivities in either serum-deprived or GDNF-treated cells.

GDNF treatment increases TH activity.

To examine if GDNF-induced changes in TH phosphorylation in BE(2)-C cells results in altered enzymatic activity, in vitro assays were performed using cell lysates. Figures 4 shows activity measurements using the water assay. A linear increase in TH activity was observed with

increasing amounts of total protein for up to 35 μg (Fig. 4a). Twenty micrograms of total protein was used in the subsequent experiments. Serum deprivation modestly increased TH activity at the 60 min time point (Fig. 4b). Consistent with the effect of GDNF on Ser31 phosphorylation, TH activity was significantly augmented at the 60 min time point compared to the serum-deprived control. To examine if the inhibition of Ser31 phosphorylation by the MEK inhibitor blocks this increase in TH activity, cells were treated with GDNF for 30 min in the presence or absence of 5 μM U0126. GDNF-mediated increases in TH activity was completely blocked in the presence of U0126 (Fig. 4c).

Fractional change of phospho- and non-phospho-TH following GDNF treatment.

Our western blot data indicated that the total amount of TH did not alter as a result of either serum deprivation or GDNF treatment, and the changes in phosphorylation detected using both the phospho- and non-phospho-specific antibodies were within the linear range. These conditions satisfy the assumptions made to derive to equations 5 and 6 and allow to calculate the fractions of phosphorylated and non-phosphorylated TH molecules (see Materials and Methods section). Since the maximal change in TH activity in response to GDNF treatment was detected at the 60 min time points, the calculations were performed using the fold changes observed at this time point. As shown in Table 1, prior to serum deprivation approximately 15% of TH molecules were phosphorylated on Ser31. Serum deprivation increased the fraction of phosphorylated TH molecules to 30%, whereas GDNF treatment resulted in 56% of all TH molecules being phosphorylated on Ser31 (Table 1).

GDNF increases TH phosphorylation, but not TH protein levels, in primary dopaminergic cells.

Since BE(2)-C cells do not synthesize dopamine, we used primary rat mesencephalic cell cultures to examine if the increased phosphorylation of TH in response to GDNF exposure leads to enhanced dopamine synthesis. To characterize the cell population, double immunostainings using a neuron-specific marker, NeuN and pan-TH antibodies were performed. Figure 6a shows that approximately 95% of the cells in these cultures were NeuN-positive, and that 2.43 +/- 0.25 percent (mean +/- SE) of neurons are TH positive in these primary cultures. These cell cultures were exposed to GDNF and cell extracts were prepared at different time points. The extracts were analyzed by western blotting using the pan-specific TH antibody. A representative western blot and the summary data compiled from five independent experiments in Figure 5b shows that GDNF treatment did not change TH immunoreactivity at any of the time points examined.

The protein samples used to examine pan-specific TH immunoreactivity in Figure 5b were further analyzed for TH phosphorylation. Figures 5c and 5d show representative western blots and summary data for phospho-Ser31 and phospho-Ser40 immunoreactivity in response to GDNF application. The immunoreactivities for both phosphorylation sites were significantly increased within minutes and remained elevated at the 3 hr time point compared with the GDNF-untreated controls (the 0 time points). The temporal change in phospho-Ser31 and phospho-Ser40 immunoreactivity was qualitatively similar for primary neurons when compared to GDNF treated BE(2)-C cells. Figure 5e shows dose-response graphs for phospho-Ser31 and phospho-Ser40 immunoreactivities in response to a 30-minute GDNF exposure. Both phospho-Ser31 and phospho-Ser40 immunoreactivities showed comparable increases with increasing GDNF concentrations up to 50 ng/mL.

Erk activity is required for Ser31, and PKA for Ser40 phosphorylation in primary neuronal cells.

As shown in Figure 3, the increases in Ser31 phosphorylation are due to GDNF-mediated Erk activation in BE(2)-C cells. To examine if GDNF-mediated Ser31 phosphorylation depends on Erk activation in dopaminergic cells, the cells were pretreated with MEK inhibitor U0126 for an hour, followed by the treatment with GDNF for 30 min. A representative western blot and the summary data compiled from three independent experiments are shown in Figure 6a. GDNF significantly enhanced the phospho-Ser31 immunoreactivity in the absence of the inhibitor. The GDNF-mediated increases in both Ser31 and Erk phosphorylations were inhibited by 5 μM U0126 (Fig. 6a, left and center). Re-probing of the membrane with the phospho-Ser40 antibody followed by the pan-specific TH antibody indicated that U0126 did not affect Ser40 phosphorylation or TH protein levels (Fig. 6a, right). As previous studies have shown that protein kinase A (PKA) can phosphorylate Ser40, we examined if GDNF-mediated Ser40 phosphorylation is altered by a PKA inhibitor, Rp-cAMPS. Phospho-Ser40 immunoreactivity was significantly decreased by 50 μM Rp-cAMPS (Fig. 6b, left). The membranes used for the western blotting of phospho-Ser40 were reprobed for phospho-Ser31, showing that phospho-Ser31 level was not affected by Rp-cAMPS (Fig. 6b, right).

GDNF increases dopamine synthesis in primary neuronal cells.

Dopamine synthesis in mesencephalic neurons was measured using the CO_2 assay. Figure 7a shows a dose-dependent increase in CO_2 release in response to exposure with different concentrations of GDNF for 30 min. To examine the effect of MEK and PKA inhibitor on

GDNF-mediated increases in dopamine synthesis, cells were preincubated with U0126 and/or Rp-cAMPS for an hour followed by the treatment with 50 ng/mL GDNF for 30 min, and CO2 assay was performed. Figure 7b shows that GDNF increases dopamine synthesis by approximately 3-fold. Rp-cAMPS treatment modestly decreased this GDNF-mediated enhanced dopamine synthesis. In contrast, U0126 significantly inhibited dopamine synthesis at two different concentrations. However, the degree of inhibition observed was similar at these two concentrations. Interestingly, treatment of cells with both U0126 and Rp-cAMPS completely blocked GDNF-stimulated dopamine synthesis.

Discussion:

The study of the effect of GDNF on dopaminergic cells has been primarily restricted to the investigation of its ability to maintain the survival of these neurons, and the usefulness of this factor as a potential therapy for diseases of the CNS (28). However, in addition to effects directly attributable to the survival of dopaminergic cells, results that appear to be related to altered dopamine biosynthesis (e.g. increases in quantal size) have been reported (2). It has been hypothesized that increases in the level of TH, the rate-limiting enzyme in the synthesis of dopamine, may underlie these effects, although experiments investigating the levels of TH following GDNF exposure have yielded contradicting results (6). As TH activity is modulated by changes in its phosphorylation state (7), we investigated the effect of GDNF on the phosphorylation of two key seryl residues. In this report, we have examined the effect of GDNF on TH phosphorylation and activity using a human neuroblastoma cell line and rat primary mesencephalic neuronal cultures. Exposure of these cells to GDNF increased TH activity, and in

the primary neurons, dopamine synthesis. This enhancement of activity was primarily caused by increased phosphorylation of TH on Ser31.

Although previous studies have reported increased phosphorylation of TH on three seryl residue (Ser19, Ser31 and Ser40) in response to treatments of cells, contribution of phosphorylation at each of these sites to TH activity remains unclear. Ser19 phosphorylation does not appear to influence TH activity in vitro (29). Phosphorylation of Ser31 and Ser40 has been reported to increase TH activity via different mechanisms. For example, Ser31 phosphorylation enhances enzymatic activity by increasing the Vmax of the enzyme (22), while phosphorylation of Ser40 is thought to enhance the rate of dissociation of catecholamines that suppresses TH activity (30). Erk1/2 are the only protein kinases, to date, that have been shown to phosphorylate Ser31 in situ both in response to depolarization and NGF treatment (12), (22). In this report, we present data to show that exposure of BE(2)-C cells to GDNF caused a rapid increase in Erk phosphorylation that lasted for up to 3hr. The phosphorylation of both Erk and Ser31 was significantly reduced by pretreatment with two different MEK inhibitors, suggesting that GDNF-induced Erk activity results in enhanced Ser31 phosphorylation leading to increased TH activity. The results presented in Table 1 show that serum deprivation increases the fraction of TH molecules, which are phosphorylated on Ser31 from 15% to 30%. This increase is accompanied by a 26% increase in TH activity. GDNF treatment increased the fraction of Ser31 phosphorylated TH molecules from 15% to 56%, a change accompanied by a 56% change in TH activity.

As observed using BE(2)-C cells, GDNF increased Ser31 phosphorylation in primary neuron cultures with comparable temporal changes (Fig 5d). GDNF treatment also increased Erk phosphorylation in these cells. The increased phosphorylation of both Erk and Ser31 was

blocked by the MEK inhibitor U0126. In contrast, the PKA inhibitor Rp-cAMPS did not block GDNF-stimulated Ser31 phosphorylation, but decreased Ser40 phosphorylation. These results are consistent with previous studies indicating that Ser31 is primarily phosphorylated by Erk in situ.

Phosphorylation of Ser40 increases TH activity, possibly via a catecholamine dissociation. Consistent with this, mutation of Ser40 to either leucine or tyrosine decreases the affinity of TH for dopamine (31). Several protein kinases including PKA, protein kinase C, cyclic GMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase II and mitogen activated protein kinase-activated protein kinase 2 (MAPKAP2) have been shown to phosphorylate this site. Our results show that GDNF exposure increases the phosphorylation of Ser40 at time points when increased TH activity was detected in both BE(2)-C cells and primary neurons. Dopamine synthesis assay in BE(2)-C cells did not yield any detectable activity at any of the time points or conditions examined. As many neuroblastoma cell lines are known to lack L-aromatic amino acid decarboxylase activity (14), which catalyzes the conversion of L-DOPA to dopamine, this would result in an overall failure of the assay. In the absence of dopamine production, the feedback inhibition of TH would not occur in BE(2)-C cells. Thus the increase in Ser40 phosphorylation we observed is unlikely to have contributed to the increases in TH enzymatic activity detected, leaving us to conclude that the increases in TH activity in response to GDNF application is most likely due to the increases in Ser31 phosphorylation in BE(2)-C cells. Consistent with this, inhibition of Ser31 phosphorylation by a MEK inhibitor completely blocked GDNF-induced increase in TH activity measured in the water assay.

In mesencephalic neuronal cells, Ser40 is also increased in response to GDNF exposure. The PKA inhibitor Rp-cAMPS decreased the phosphorylation of Ser40 without changing the

Ser31 phosphorylation. This indicates that GDNF-mediated activation of PKA is primarily responsible of Ser40 phosphorylation. At present, it is not known how GDNF can stimulate synthesis of cAMP and activity of PKA. One possibility is that binding of GDNF to GFRα-Ret receptor stimulates phospholipase Cγ activity leading to increases in intracellular calcium (32). Increases in intracellular calcium can stimulate the activity of calcium-sensitive adenylyl cyclase and cAMP levels (33).

Unlike the BE(2)-C cells, mesencephalic neurons show increased dopamine synthesis in response to GDNF exposure. A 30-min exposure to GDNF increased dopamine synthesis by approximately 3-fold. Both 5 and 50 μM U0126 inhibited dopamine synthesis to the same degree. When a combination of U0126 and Rp-cAMPS was utilized, a complete blockade of GDNF-mediated enhanced dopamine synthesis was observed, returning the activity to the basal levels. In addition to a direct activation of TH via increased phosphorylation, GDNF can indirectly increase TH activity. For example, it has been reported that GDNF increases GTP-cyclohydrolase I activity (a key enzyme in BH4 synthesis) and BH4 levels in primary dopamine neurons (34). GTP-cyclohydrolase I activation was observed at the 24 hr and 48 hr, but not at 6 hr, following GDNF treatment. This suggest that increases in GTP-cyclohydrolase I and BH4 levels can contribute to TH activity at longer time points, and may not have contributed to the changes in TH activity observed in the present study.

Can chronic GDNF administration elevate dopamine levels? It has been reported that chronic GDNF treatment decreases TH protein level (6). In our findings, TH activity is initially increased followed by a steady decline over time. The mechanism(s) for this decline in activity over time in the continued presence of GDNF is not known at present. Several mechanisms are plausible including receptor desensitization and activation of a protein phosphatase. Our

preliminary examination shows that chronic treatment with GDNF causes a decrease in Ser19 phosphorylation (data not shown) consistent with a phosphatase activation. In addition to the effect of GDNF on TH phosphorylation and dopamine synthesis, it also acts as a potent survival factor and causes collateral sprouting of dopamine cells. Even if chronic GDNF treatments may not increase TH activity, its effect on dopamine cell survival and collateral sprouting may result in an overall increase in dopamine levels. This, however, remains to be demonstrated in animal models.

In conclusion, the present study shows that in addition to its survival effects, GDNF can increase the activity of the rate-limiting enzyme of dopamine biosynthesis, and suggests that impaired dopaminergic function can be acutely altered by GDNF treatment. Future studies will examine if administration of GDNF can alter the phosphorylation and activity of TH in vivo, and offer beneficial effects for neurological dysfunctions associated with decreased dopamine levels.

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Figure Legends:

Figure 1

Differentiation of BE(2)-C human neuroblastoma cells and characterization of TH antibodies. **a.** Morphology of the cells before and after the treatment with all trans-retinoic acid (RA) for 6 days as seen using a phase-contrast microscope. RA treatment increased the number and length of processes, and the immunoreactivities for pan-specific TH and synaptophysin. **b.** RA treatment for 6 days (n=3) increased TH activity as assessed using the water assay. *, $P < 0.05$. **c.** Immunoblotting of bacterially expressed bovine TH protein using pan-, phosphorylation- and non-phosphorylation-specific TH antibodies. The phosphorylation specific antibodies did not bind to bacterially expressed TH. **d.** Immunoblotting for pan-specific and phosphorylation-specific TH antibodies. Lane 1: bovine adrenal gland, lane 2: BE(2)-C, lane 3: Jurkat T-cell and lane 4: pre-absorbed phosphorylation-specific antibody.

Figure 2

GDNF exposure alters TH phosphorylation in BE(2)-C cells. Optical density of immunoreactive bands at each time point is plotted as fold-change (mean +/- SE) compared to the density at zero time point (n=3). Pictures of representative western blots are shown. **a.** Pan-specific (total) TH, **b.** TH phospho-Ser40, **c.** TH nonphospho-Ser40, **d.** TH phospho-Ser31 and **e.** TH nonphospho-Ser31. *, $P < 0.05$ between GDNF-treated and -untreated groups. +, $P < 0.05$ compared with zero time point controls.

Figure 3

Increases in Ser31 phosphorylation in response to GDNF exposure is caused by enhanced Erk activity in BE(2)-C cells. **a.** GDNF treatment significantly increases the immunoreactivity for phospho-Erk1/2 (n=3). **b.** Phospho-Ser31 and phospho-Erk immunoreactivities are enhanced with increasing concentrations of GDNF (n=2). Immunoreactivity signals obtained from untreated samples were used to calculate the fold-changes at each GDNF concentration. **c.** Effect of MEK inhibitors on phospho-Ser31, phospho-Erk, pan-TH, and phospho-Ser40 immunoreactivities (n=3). **d.** Effect of LY294002 on phospho-Ser31 and phospho-Erk immunoreactivities (n=3). Control samples were obtained from cells not treated with either GDNF, or MEK inhibitors. *, $P < 0.05$ between GDNF-treated and -untreated groups. +, $P < 0.05$ compared with zero time point controls.

Figure 4

Measurements of TH enzymatic activity in BE(2)-C cells using the water assay. **a.** Radioactive water production increases in a linear fashion with respect to protein amount in the assay mixture (n=2). **b.** TH activity increases with GDNF treatment (n = 3). TH activity was plotted as fold-changes compared with the zero time point controls. *, $P < 0.05$ between GDNF-treated and –untreated groups. +, $P < 0.05$ compared with the zero time point controls. **c.** GDNF-mediated increases in TH activity are blocked by treatment with the Erk inhibitor U0126 (n=3). Control samples were obtained from cells untreated with U0126 or GDNF. *, $P < 0.05$ between U0126-treated and -untreated groups. +, $P < 0.05$ between GDNF-treated and -untreated groups.

Figure 5

GDNF exposure increases TH phosphorylation, but not TH protein levels, in mesencephalic dopaminergic cells. **a.** Pictures of cultured mesencephalic dopaminergic cells stained with the neuron-specific marker NeuN and NeuN **+** pan-specific TH antibodies. GDNF treatment did not alter the immunoreactivity of **b.** Pan-specific TH., but significantly increased the immunoreactivities for **c.** TH phospho-Ser40. and **d.** TH phospho-Ser31. Optical density of immunoreactive bands at each time point is plotted as fold-change (mean +/- SE) compared to the zero time point (n=5). Pictures of representative western blots are shown. +, $P < 0.05$ compared with zero time point controls. **e.** Phospho-Ser31 and phospho-Ser40 immunoreactivities increase with increasing GDNF concentrations. The cells were treated with GDNF for 30 min (n=2). The graph was plotted as fold-changes of immunoreactivity compared with samples obtained from the untreated cells.

Figure 6

Increases in Ser31 and Ser40 phosphorylation in response to GDNF exposure is mediated via Erk and PKA, respectively, in dopaminergic neurons. Optical density of immunoreactive bands in each time point is plotted as fold-change (mean +/- SE) compared with the zero time point (n=5). Pictures of representative blots are shown in the upper rows. **a.** U0126 treatment blocked the GDNF-mediated increases in phospho-Ser31 and phospho-Erk, but not phospho-Ser40, immunoreactivities. **d.** Rp-cAMPS treatment reduced the GDNF-mediated increase in

phospho-Ser40, but not phospho-Ser31, immunoreactivity. *, $P < 0.05$ between inhibitor-treated and -untreated groups. +, $P < 0.05$ between GDNF-treated and -untreated groups.

Figure 7

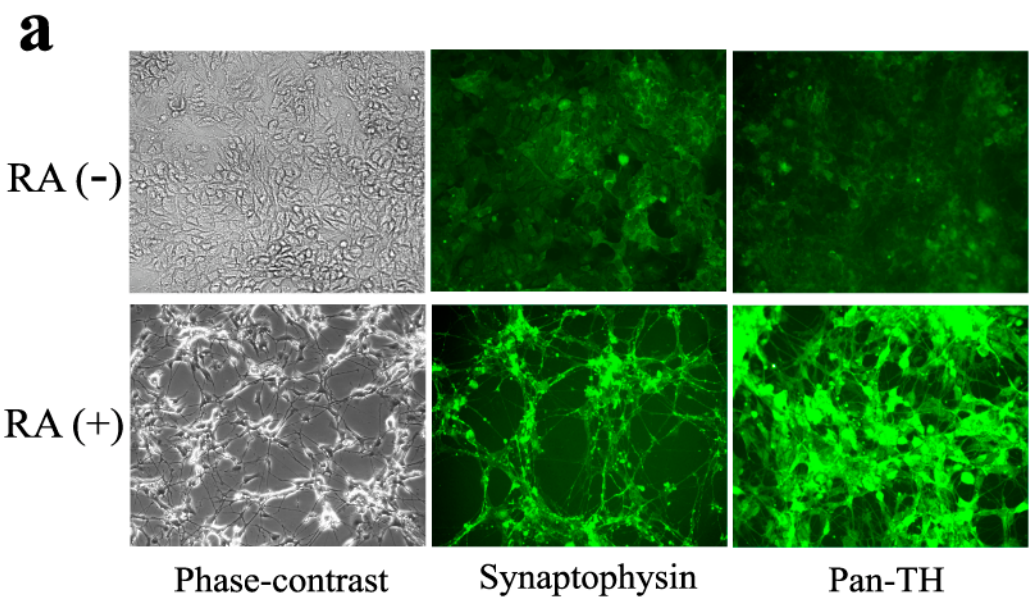
Measurement of dopamine synthesis in mesencephalic cell cultures using the CO_2 assay. Results are plotted as the mean +/- SE. **a.** Dopamine synthesis, as measured by radioactive CO_2 production, is enhanced with increasing concentrations of GDNF. Cells were treated with GDNF for 30 min (n=3 per concentration). **b.** Inhibition of Erk activity by UO126 significantly reduced the GDNF-mediated increase in dopamine synthesis. The combination of Rp-cAMPS and U0126 blocked the GDNF effect on dopamine synthesis, resulting in CO_2 production comparable to untreated controls (n=3). *, $P < 0.05$ between inhibitor-treated and -untreated groups. +, $P < 0.05$ between GDNF-treated and -untreated groups.

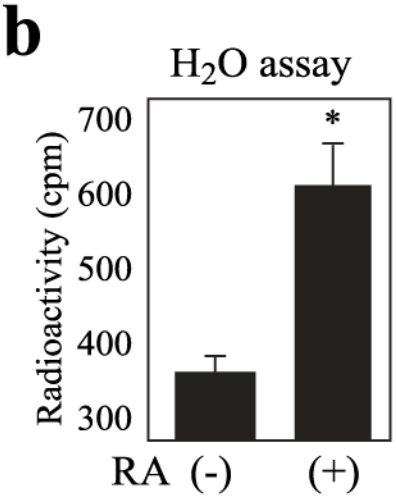
Table 1 **Fractional change of phosphorylated TH and enzymatic activity following 60 min of serum deprivation with or without GDNF**

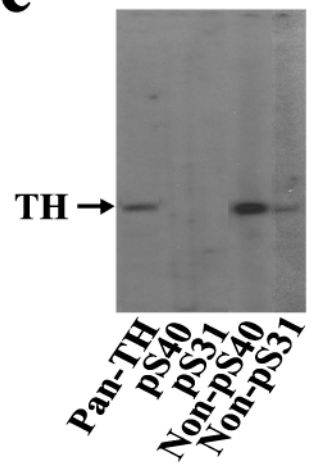
	Fold-change		Calcurated values (%)				TH activity
	x	y	P_0	N_0	Pt	Nt	(%)
pSer31 without GDNF	2.02	0.82	15	85	30	70	26
pSer31 with GDNF	3.85	0.51	15	85	56	44	56

x, y: fold-change of phospho- and non-phospho-TH, respectively
P0, Pt: fraction of phospho-TH at the 0 and 60 min time points, respectively
N0, Nt: fraction of non-phospho-TH at the 0 and 60 min time points, respectively

Figure 1


a
RA (-)
RA (+)
Phase-contrast
Synaptophysin
Pan-TH


b
H2O assay
Radioactivity (cpm)
700
600
500
400
300
*
RA (-)
(+)


c
TH
Pan-TH
pS40
pS31
Non-pS40
Non-pS31

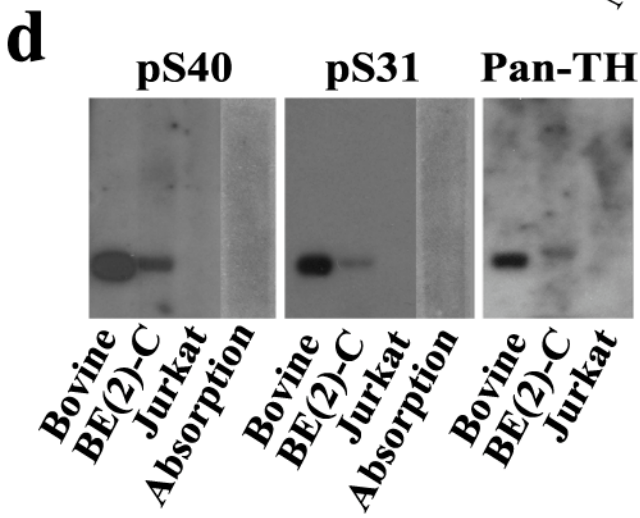

d
pS40
pS31
Pan-TH
Bovine
BE(2)-C
Jurkat
Absorption
Bovine
BE(2)-C
Jurkat
Absorption
Bovine
BE(2)-C
Jurkat

Figure 2

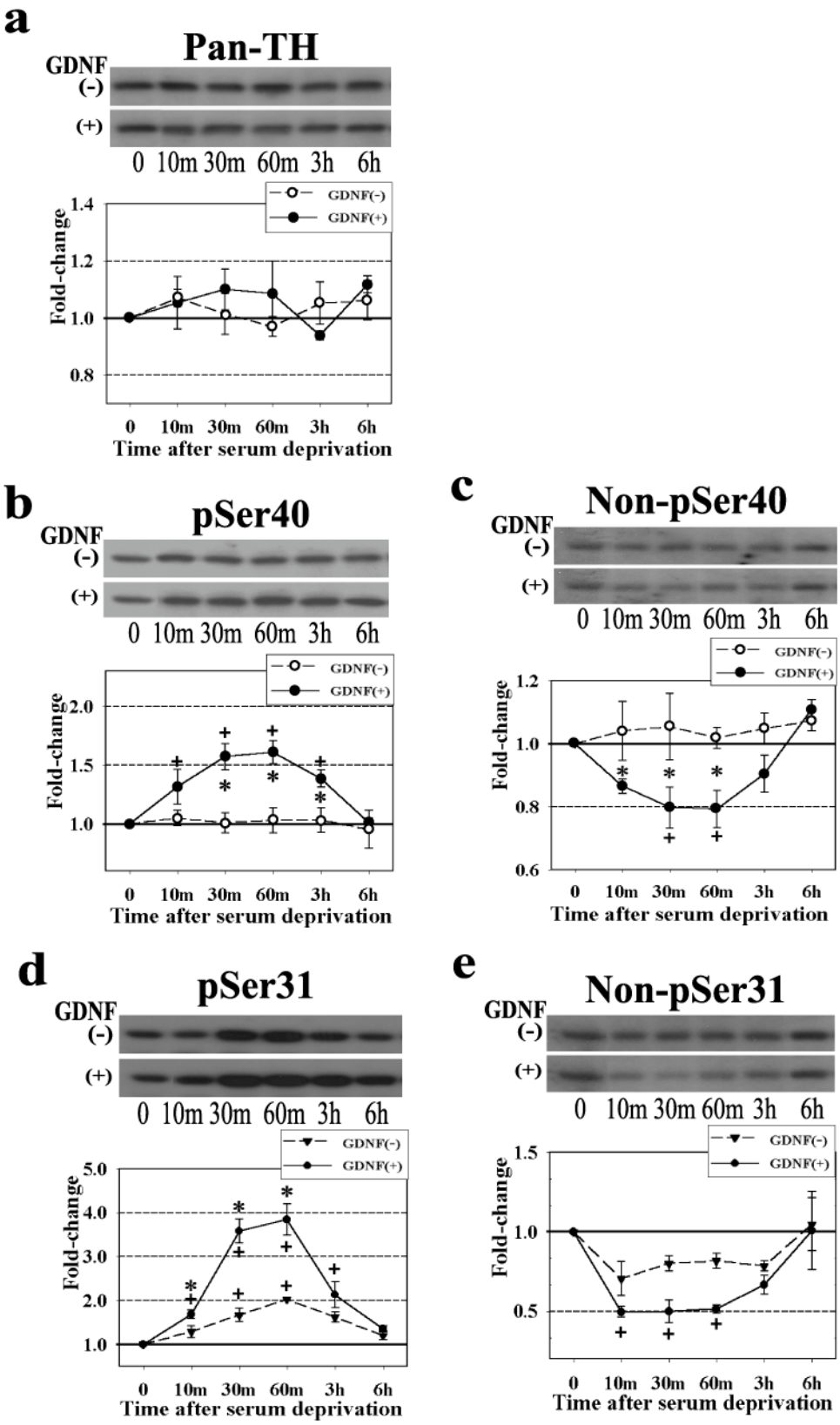

a
Pan-TH
GDNF
(-)
(+)
0 10m 30m 60m 3h 6h
GDNF(-)
GDNF(+)
Fold-change
Time after serum deprivation
b
pSer40
c
Non-pSer40
d
pSer31
e
Non-pSer31

Figure 3

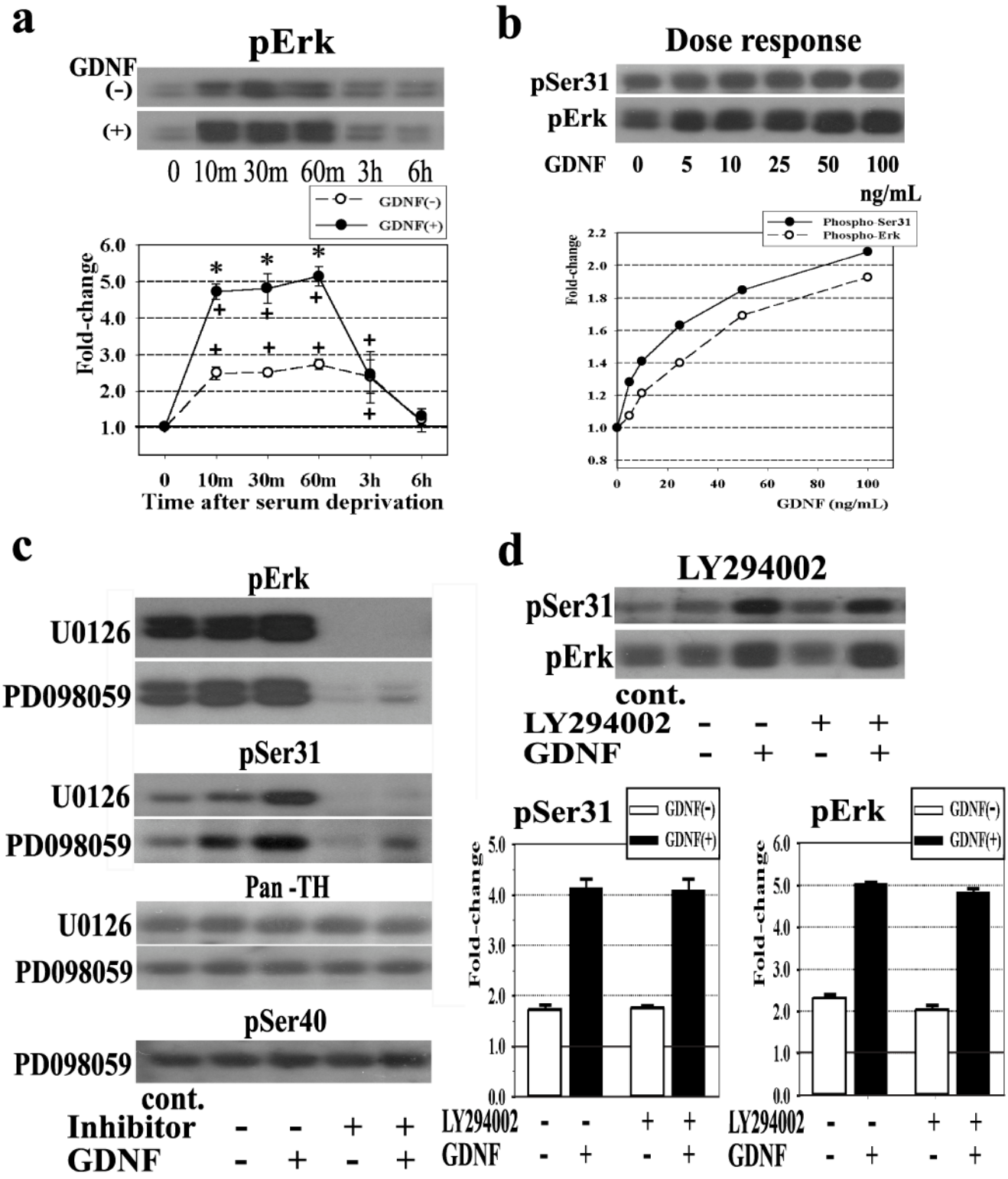

a
pErk
GDNF
(-)
(+)
0 10m 30m 60m 3h 6h
GDNF(-)
GDNF(+)
Fold-change
Time after serum deprivation
b
Dose response
pSer31
pErk
GDNF 0 5 10 25 50 100
ng/mL
Phospho-Ser31
Phospho-Erk
Fold-change
GDNF (ng/mL)
c
pErk
U0126
PD098059
pSer31
Pan -TH
pSer40
cont.
Inhibitor
GDNF
d
LY294002
pSer31
pErk
cont.
LY294002
GDNF
pSer31
pErk
GDNF(-)
GDNF(+)
Fold-change

Figure 4

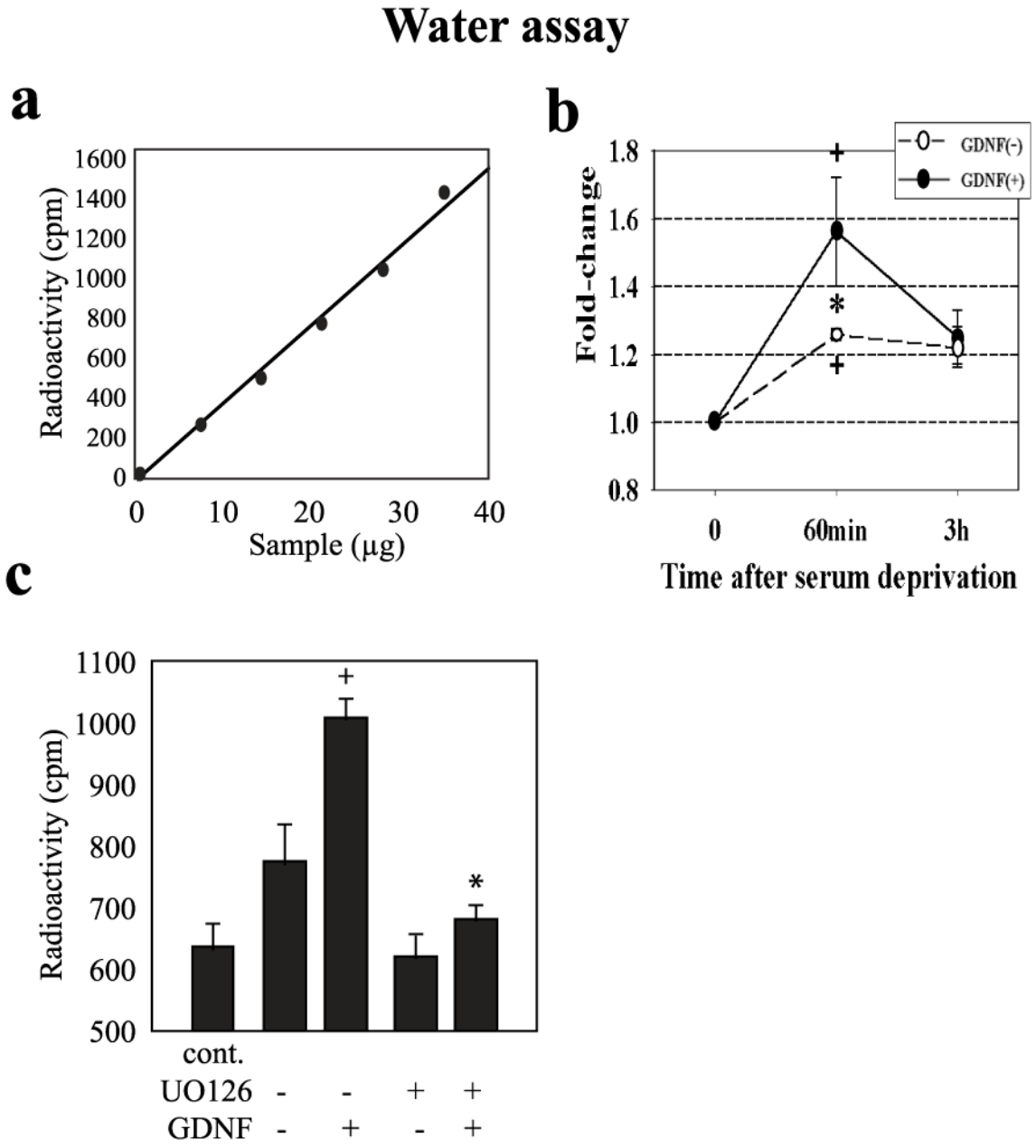

Water assay
a
Radioactivity (cpm)
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
Sample (μg)
b
Fold-change
1.8
1.6
1.4
1.2
1.0
0.8
GDNF(-)
GDNF(+)
*
+
+
0
60min
3h
Time after serum deprivation
c
Radioactivity (cpm)
1100
1000
900
800
700
600
500
+
*
cont.
UO126 - - + +
GDNF - + - +

Figure 5

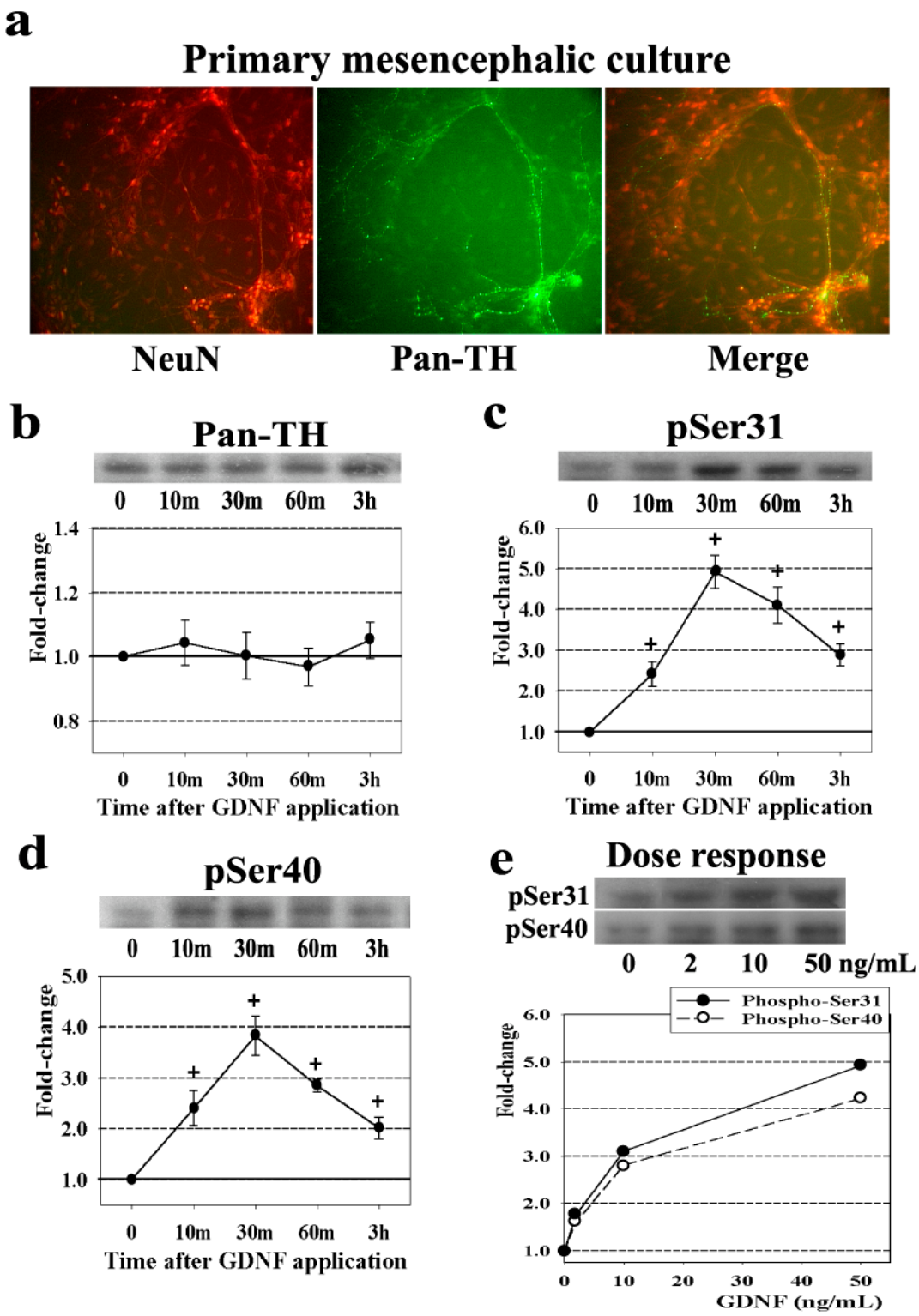

a
Primary mesencephalic culture
NeuN
Pan-TH
Merge
b
Pan-TH
0 10m 30m 60m 3h
Fold-change
1.4
1.2
1.0
0.8
Time after GDNF application
c
pSer31
0 10m 30m 60m 3h
Fold-change
6.0
5.0
4.0
3.0
2.0
1.0
Time after GDNF application
d
pSer40
0 10m 30m 60m 3h
Fold-change
5.0
4.0
3.0
2.0
1.0
Time after GDNF application
e
Dose response
pSer31
pSer40
0 2 10 50 ng/mL
Phospho-Ser31
Phospho-Ser40
Fold-change
6.0
5.0
4.0
3.0
2.0
1.0
0 10 20 30 40 50
GDNF (ng/mL)

Figure 6

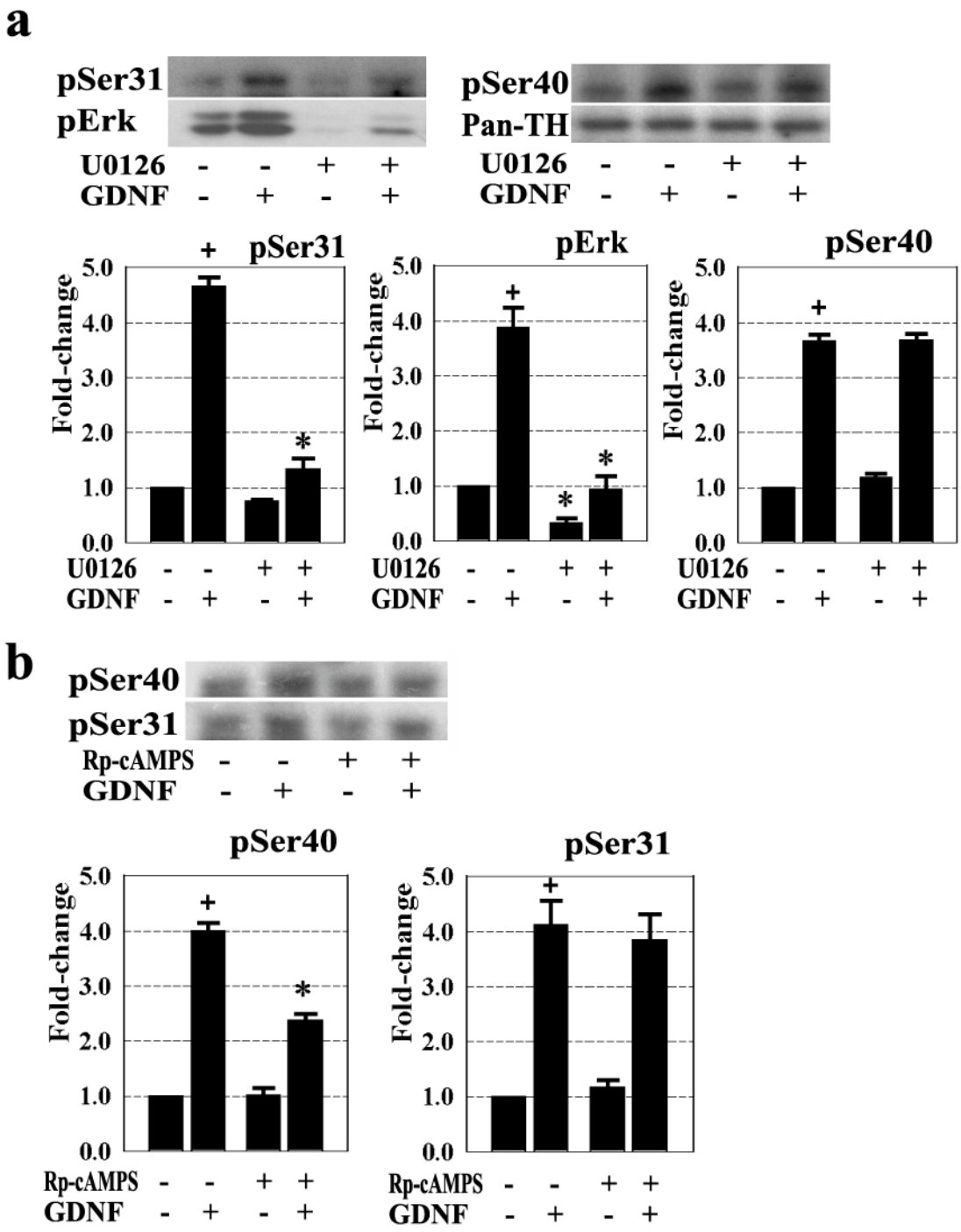
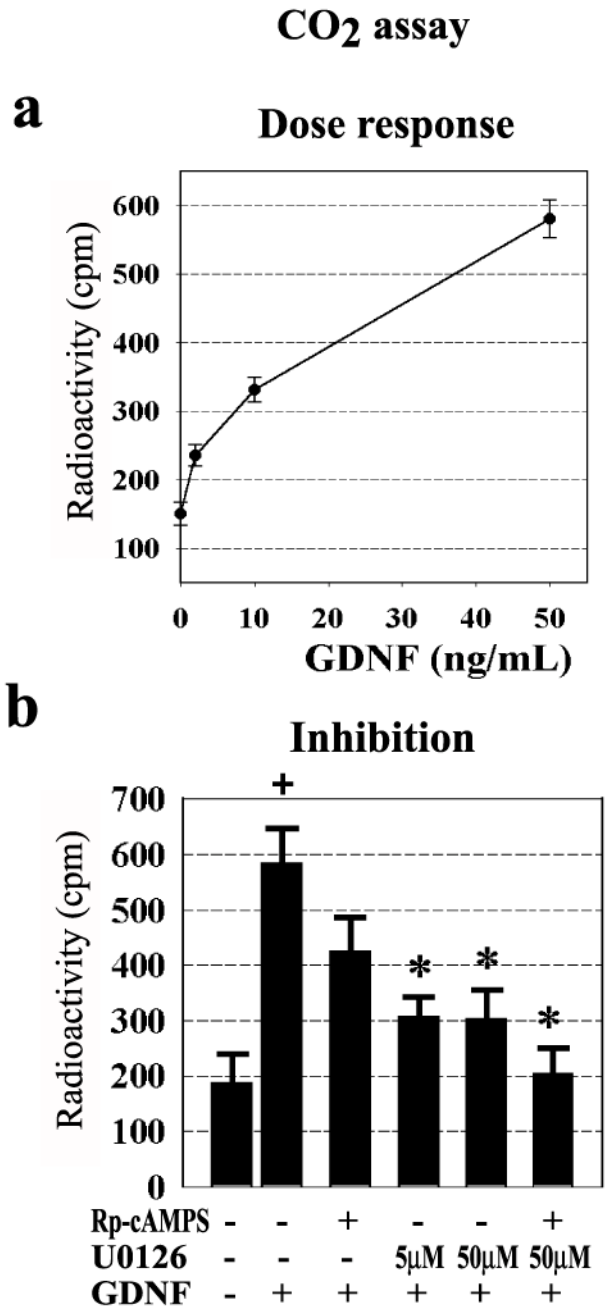

a
pSer31
pErk
U0126 - - + +
GDNF - + - +
pSer40
Pan-TH
U0126 - - + +
GDNF - + - +
pSer31
pErk
pSer40
Fold-change
5.0
4.0
3.0
2.0
1.0
0.0
+
*
U0126 - - + +
GDNF - + - +
b
pSer40
pSer31
Rp-cAMPS - - + +
GDNF - + - +
pSer40
pSer31
Fold-change
Rp-cAMPS - - + +
GDNF - + - +

Figure 7


CO2 assay
a
Dose response
Radioactivity (cpm)
600
500
400
300
200
100
0
10
20
30
40
50
GDNF (ng/mL)
b
Inhibition
Radioactivity (cpm)
700
600
500
400
300
200
100
0
+
*
*
*
Rp-cAMPS - - + - - +
U0126 - - - 5μM 50μM 50μM
GDNF - + + + + +