Persistent TrkA Activity is Necessary to Maintain Transcription in
Neuronally Differentiated PC12 cells

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SUMMARY

Neurotrophins are required for the differentiation and survival of several different neuronal subpopulations in the developing nervous system. The PC12 cell line responds to nerve growth factor [NGF] by withdrawing from the cell cycle and acquiring a sympathetic neuron-like phenotype. Previous studies have shown that the activation kinetics of the NGF receptor, TrkA, and downstream protein kinases appear rapid and seemingly transient after NGF treatment of naive PC12 cells. However, maintenance of the neuronal phenotype and survival of differentiated PC12 cells under serum-free conditions require constant NGF exposure. In this study we have addressed the mechanisms that NGF uses to maintain neuronal PC12 cells. We show that TrkA remains phosphorylated at a basal level throughout differentiation of the PC12 cells. The phospho-TrkA levels in the differentiated PC12 cells were diminished by both complete NGF-withdrawal and pharmacological inhibition of Trk kinase activity. Intracellular sequestration of the majority of TrkA molecules (both phosphorylated and non-phosphorylated TrkA) and persistent dephosphorylation of the small pool of cell surface TrkA renders the persistent phospho-TrkA signal in the differentiated PC12 cells resistant to partial NGF withdrawal as well as exposure to additional NGF. NGF regulated both Erk 1/2 and Akt activity in the differentiated PC12 cells via sustained TrkA activity. Moreover, analysis of transcription using AP1-, SRE-, and CRE-Luc reporter constructs showed that NGF regulated these promoters through TrkA activity in differentiated PC12 cells. Interestingly, the initial response of the CRE-promoter to NGF
was delayed, becoming Trk-dependent well beyond the peaks in TrkA and downstream protein kinase signal transduction.

**Keywords**: Nerve growth factor, neuronal differentiation, PC12, transcription, CREB, Trk.
INTRODUCTION

During development, nerve growth factor (NGF)\(^1\) has profound effects on the differentiation and survival of subsets of neurons in the peripheral and central nervous systems (1). These effects are largely mediated through activation of the TrkA receptor, which initiates a cascade of signaling events that include activation of several downstream protein kinases and transcription factors (2). These early events are thought to generate the long-term changes in gene expression needed for acquisition of a mature neuronal phenotype. Mature or adult neurons are also exposed to and responsive to neurotrophins like NGF, but their biological responses may be quite distinct from those of an immature neuron. For example, developing nociceptive neurons in the dorsal root ganglion require NGF for survival (3). NGF alters neurite outgrowth, gene induction and the responsiveness of adult nociceptive neurons to some stimuli but it is apparently not necessary for their survival (4-6). Much effort has been devoted towards understanding how NGF and other neurotrophins bring about their biological effects in both the developing and adult nervous systems. Most studies of neurotrophin signal transduction have focused on how these ligands activate intracellular signals. However, activity of the intracellular mediators that the neurotrophins use must somehow be maintained to generate their lasting biological effects.

\(^1\)Abbreviations: NGF, nerve growth factor; Erk 1/2, extracellular-regulated kinases; EGF, epidermal growth factor; PI3K, phosphatidylinositol-3 kinase; CRE, cyclic AMP response element; SRE, serum response element; AP-1, activating protein 1; CREB, cAMP response element binding protein; DMEM, Dulbecco’s modified essential medium; DMEB, DMEM supplemented with bovine serum albumin; PMA, phorbol 12-myristate 13-acetate; dbcAMP, dibutyryl cAMP; RLU, relative light units; TBST, Tris-buffered saline plus 0.2 % Tween-20; MTT, methylthiazolotetrazolium; SRF, serum response factor; MEK-1, mitogen-activated protein kinase kinase 1 (MAPKK1); JNK, c-jun N-terminal kinase; p38\(^{MAPK}\), 38 kDa mitogen-activated protein kinase; p75\(^{LNT1}\), 75 kDa low affinity neurotrophin receptor; and CBP, CREB binding protein.
The NGF-responsive PC12 cell line has been used extensively for analysis of neurotrophin signal transduction. NGF induces the differentiation of PC12 cells into a sympathetic neuron-like phenotype (7). Both survival and phenotypic maintenance of the differentiated or 'neuronal' PC12 cells require continual exposure to NGF (8). Previous studies, using either activating and dominant negative signaling proteins or pharmacological inhibitors, have argued that sustained activity of Erk 1/2 and/or PI3K is required to maintain survival of differentiated PC12 cells (2,9,10). However, there are exceedingly few examples where the activity of intracellular signal transduction pathways in differentiated PC12 cells has been directly demonstrated. Even when NGF is continually present and its receptors are available to bind ligand, activation of TrkA appears to be a relatively short-lived event in the PC12 cells. TrkA activity falls dramatically over a few hours time following the large peak activity at 5 min after initial NGF exposure (11). Comparing the kinetics of Erk 1/2 activation in PC12 cells treated with NGF versus mitogenic stimuli has led to the conclusion that prolonged activation of Erk 1/2 drives NGF-dependent differentiation of PC12 cells (12,13). NGF-dependent activation of Erk 1/2 is initially activated by Ras and then prolonged activity is maintained by Rap 1. Mitogenic stimuli such as EGF only stimulate Ras activity in the PC12 cells so Erk 1/2 activity is not maintained for hours as seen after NGF stimulation (14). However, since the increased activity of Erk 1/2 seen after NGF treatment takes place over the course of hours, this cannot explain the means that NGF uses to drive neuronal differentiation over the days of ligand exposure needed for this phenotypic transition (8,15).
Here, we show that the NGF receptor, TrkA, remains phosphorylated on residues that correspond to the activated receptor in PC12 cells treated with NGF for 1-7 days. As long as NGF is present, TrkA activity is greater than that seen in naive PC12 cells. Continual NGF exposure also sustains a basal, homeostatic level of Erk 1/2 and Akt phosphorylation on activating residues and this requires persistent TrkA activity. Thus, NGF maintains a basal level of activity in both its receptor and downstream signaling pathways well beyond the rapid activation kinetics that have been previously ascribed to neurotrophin signal transduction. Using stably transfected PC12 cell lines, we further show that this basal TrkA activity regulates transcription through CRE, SRE, and AP-1 promoter elements in the differentiated PC12 cells. Although previous studies have shown that the CRE binding protein [CREB] is rapidly activated by NGF treatment in naive PC12 cells (16), we show that transcription from a CRE reporter construct does not become TrkA-dependent until well beyond the initial burst of TrkA activity. This argues that the new basal level of TrkA tyrosine kinase activity that is reached after the first day of NGF treatment is biologically relevant for both activating and maintaining gene expression in the differentiating PC12 cells.
EXPERIMENTAL PROCEDURES

Cell culture – PC12 cells were maintained in Dulbecco’s modified Eagle’s medium [DMEM] containing 6% horse serum and 6% bovine calf serum (Hyclone) at 7% CO₂, 37°C. For NGF treatment, PC12 cells were plated on collagen-coated dishes in complete media for at least 6 h and then switched to serum-free conditions consisting of DMEM containing 0.1% bovine serum albumin [DMEB] supplemented with 50 ng/ml NGF (Harlan Bioscience). Medium and NGF were replenished every 2 days. Medium changes were performed at least 24 h prior to any experiments in NGF treated cells to ensure that a basal state of signaling had been reached. For pharmacological inhibition of Trk, MEK1, or PI3K activity in naive cells, cultures were pretreated with 200 nM K252a (Calbiochem), 50 μM PD98059 (Biomol), or 50 μM LY294002 (Biomol), respectively, for 30 min before addition of NGF; in PC12 cells that had been treated with NGF for ≥ 24 h, inhibitors were added directly to culture media. For control, cells were treated with an equal volume of vehicle (DMSO). The stably transfected PC12 clones described below were treated in the same fashion.

Transfection and characterization of transcriptional activity – PC12 cells were co-transfected with the pAP1-Luc, pCRE-Luc, or pSRE-Luc (Stratagene) plus pSV3-neo plasmids using Lipofectamineplus (Invitrogen, Inc.). Stable clones were selected in 400 μg/ml G418 and maintained in 100 μg/ml G418 as previously described (17,18). Stable clones were chosen for further experiments based upon an appropriate induction of luciferase activity using known activators of these reporter elements (see below). AP1-Luc and SRE-Luc clones were treated with 1 μM phorbol 12-myristate 13-acetate [PMA] (Sigma). CRE-Luc clones were treated with 1 μM dibutyryl-cAMP [db-cAMP]
(Sigma) for 5 h. Reporter activity was assessed by luminometry using a commercial luciferase assay kit (Promega). The protein concentration of each lysate was determined by Bradford assay and the luciferase activities, measured in relative light unit values [RLU], were normalized for protein content in each sample.

In the naive clones, accumulation of luciferase activity after 5 h of NGF stimulation was used as a measure of AP1-, SRE-, and CRE-dependent transcription. Analysis of relative transcriptional activity in differentiated clones required consideration of decay of both luciferase mRNA and protein. 20 h proved the optimal duration of anti-NGF or K252a treatment for assessing relative transcriptional activity from the AP1-, SRE-, and CRE- promoter elements.

**Titration of neutralizing anti-NGF antibody and NGF withdrawal**  – Neutralizing anti-NGF antibody was titrated in PC12 cells using phosphorylation of TrkA at 5 min as a biological measure of NGF levels. For this, 50 ng/ml NGF was incubated in DMEB with serial dilutions of rabbit anti-NGF antibody for 20 min. Naive PC12 cells were then treated with the NGF/anti-NGF antibody mixtures for 5 min, washed twice in ice cold PBS, and processed for immunoblotting (see below). For NGF neutralization in NGF-treated cultures, PC12 cell cultures were treated with 50 ng/ml NGF for the indicated durations and then the anti-NGF antibody was added directly to culture medium as indicated in the results section.

**Immunoblotting**  – Cells were washed twice in ice cold PBS, and then lysed in lysis buffer (20 mM Tris-Cl [pH 7.4], 150 mM NaCl, 1 % Triton X-100, 20 mM NaF, 2 mM para-nitrophenol phosphate, 1 μg/ml leupeptin, 2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride) (17,18). After clearing at 13,000 rpm for 15 min, lysates
were normalized for protein content using Bradford assay (Biorad). Equal amounts of protein were separated on denaturing polyacrylamide gels and then transferred to PVDF membranes. Membranes were blocked in TBST (10 mM Tris [pH 7.4], 150 mM NaCl, 0.1% Tween-20) containing 5 % non-fat dry milk for 1 h followed by an overnight incubation with primary antibodies diluted at 4°C. Primary antibodies were diluted in blocking buffer as follows: rabbit anti-Trk\textsuperscript{PY490} (1:1000; Cell Signaling Technology), rabbit anti-TrkA-ex (1:10,000 (19)); rabbit anti-active MAPK (1:5000; Promega), rabbit anti-Erk 1/2 (1:10,000; Santa Cruz Biotechnology), rabbit anti-Akt\textsuperscript{PS473} (1:1000; Cell Signaling Technology), rabbit anti-Akt (1:1000; Cell Signaling Technology), and rabbit anti-eIF5 (1:1000; Santa Cruz Biotechnology). The membranes were then washed in TBST and incubated for 60 min with horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:2000; Cell Signaling Technology). Reactive bands were detected by using enhanced chemiluminescence per the manufacturer's instructions (ECL plus, Amersham).

**Cell surface biotinylation** – Isolation of cell surface proteins was performed essentially as previously described with minor modifications (20). Briefly, naive or differentiated PC12 cells were rinsed three times with ice cold PBS and then incubated with either PBS alone (non-biotinylated controls) or PBS containing 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce) for 2 h at 4°C. The biotin solution was removed and replaced with ice cold DMEM (containing glutamine) for 10 min at 4°C. The cells were gently rinsed twice with ice cold PBS and then lysed as described above. The lysates were incubated on ice for 10 min and then cleared by centrifugation at 13,000 rpm, 4°C for 15 min. Biotinylated proteins were precipitated by adding 40 µl streptavidin agarose...
beads [SA] (Molecular Probes) and incubated overnight at 4°C. The SA beads were pelleted by centrifugation and washed five times with 1 ml ice cold PBS prior to resuspension in 2x Laemli sample buffer. Precipitated proteins were then resolved on 8 % SDS/PAGE gels and then processed for immunoblotting as described above. Blots were reprobed with a rabbit anti-eIF5 antibody to ensure that the proteins detected in the SA precipitates did not arise from inadvertent cell lysis during the biotinylation procedure.

**Survival assays** – Survival assays were performed 24-48 h after NGF withdrawal using the methylthiazoletetrazolium [MTT] assay as previously described with some modifications (21,22). Briefly, MTT reagent (Sigma) was added to cultures at a final concentration of 0.5 mg/ml and the cultures were placed at 37°C, 7 % CO₂ for 2 h. Media was then removed and the insoluble blue reaction product was solubilized in DMSO. Optical densities of the medium containing solubilized MTT reagent were measured at 570 nm using a spectrophotometer.
RESULTS

*TrkA remains activated over the course of neuronal differentiation in PC12 cells*

Previous studies have shown that the initial stimulation of TrkA phosphorylation and activation of downstream signaling pathways in PC12 cells exposed to NGF is rapid but transient despite continued NGF exposure (11,23). Studies in our PC12 cell isolate showed similar rapid activation kinetics of TrkA and downstream signaling cascades (Figure 1). Activation of TrkA, determined by phosphorylation of tyrosine 490 \([\text{Trk}^{\text{PY490}}]\), peaked within 5 min of NGF addition and fell dramatically over the subsequent 15 h (Figure 1A). Activation of Erk 1/2, detected using an antibody specific for Erk phosphorylated on threonine 183 and tyrosine 185 \([\text{Erk 1/2}^{\text{PT183/PY185}}]\), followed a similar time course (Figure 1B). Consistent with prior reports, Erk 1/2\(^{\text{PT183/PY185}}\) stays at relatively high levels up to 6 h after NGF treatment (12,13,24). Similarly, levels of Akt phosphorylated at serine 473 \([\text{Akt}^{\text{PS473}}]\) remained elevated for up to 15 h after NGF treatment (Figure 1C) but fell to low levels by 24 h [data not shown].

Despite the seemingly transient increase in TrkA activity above, we were able to detect levels of phosphorylated TrkA at 1, 2, and even 7 days after the initial NGF exposure and these signals were clearly distinguished from any \([\text{Trk}^{\text{PY490}}]\) levels in unstimulated PC12 cells (Figure 2A, lanes 5-8). Since the \([\text{Trk}^{\text{PY490}}]\) signal was comparable at all of the time points after 24 h this suggests that TrkA reaches a basal level of activity that persists over the entire course of neuronal differentiation in the PC12 cells. Total TrkA levels, detected with antibodies that recognize both
phosphorylated and non-phosphorylated TrkA, remained relatively unchanged over the course of differentiation (Figure 2A, lower panel). Exposure of 7 day NGF-differentiated PC12 cells to an additional 50 ng/ml of NGF for 5 min, which is the initial time point of peak TrkA activity seen in NGF-treated naive PC12 cells (see Figure 1), did not increase the levels of phospho-TrkA detected (Figure 2A, lanes 7-8). Supplementing the 7 day NGF-differentiated cultures with an additional 50 ng/ml NGF for longer durations did not increase phospho-TrkA levels (10 min to 6 h; data not shown), arguing that TrkA does not exhibit altered activation kinetics in the differentiated PC12 cells. Total TrkA levels did not change significantly over the course of NGF differentiation in this experiment, indicating that only a fraction of TrkA receptors are activated at any one time in the differentiated PC12 cells. The persistent Trk$^{PY490}$ signal in the 7 day differentiated PC12 cells is approximately 15 fold less than that seen upon initial peak activation of TrkA in naive PC12 cells (Figure 2B).

To determine whether the Trk$^{PY490}$ signal that we had detected in the differentiated PC12 cells above is not due to non-specific recognition of unphosphorylated TrkA by the anti-Trk$^{PY490}$ antibody, we treated differentiated PC12 cells with the tyrosine kinase inhibitor K252a. In naive PC12 cells, 200 nM K252a completely blocks NGF-dependent activation of TrkA and downstream signaling cascades (25-27). Treatment of 7 day differentiated PC12 cells with 200 nM K252a diminished the Trk$^{PY490}$ signal within as little as 2 h (Figure 2B, lane 9 and Figure 5A, lane 2). Thus, the Trk$^{PY490}$ signal that we detected in the differentiated PC12 cells represents a sustained activation of the TrkA kinase.
Persistent TrkA phosphorylation is NGF-dependent in differentiated PC12 cells

To determine whether the TrkA phosphorylation that we observed in the differentiated PC12 cells is NGF-dependent, we used a neutralizing anti-NGF antibody to withdraw NGF from 7 day differentiated PC12 cells. The anti-NGF antibody was titrated in naive PC12 cells to determine the amount required to effectively neutralize 50 ng/ml NGF. 1:500 dilution of anti-NGF antibody was sufficient to completely block NGF-induced tyrosine phosphorylation of TrkA (Figure 3A, lane 1). 1:2500 dilution of anti-NGF antibody partially neutralized the 50 ng/ml NGF solution (Figure 3A, lane 6 top panel) decreasing TrkPY490 to approximate levels seen with 5 ng/ml NGF treatment (Figure 3A, lane 4 lower panel). In the differentiated PC12 cells, anti-NGF antibody decreased TrkPY490 levels at 2 h and completely depleted the TrkPY490 signal by 24 h (Figure 3B, lanes 3 and 6). The 1:2500 anti-NGF antibody treatment did not cause a discernable change in the level of TrkPY490 in the differentiated cells even after long-term antibody treatment (Figure 3B, lanes 2 and 5) despite that the 1:2500 dilution of anti-NGF caused approximately 80 % reduction of TrkA activation in the naive PC12 cells. Taken together with the NGF supplementation experiment shown in Figure 2, these data suggest that basal TrkA phosphorylation in differentiated PC12 cells is less susceptible to varying ligand concentrations than is initial activation of TrkA in naive cells.

The majority of TrkA receptors are intracellular in differentiated PC12 cells

The relative resistance of the basal TrkPY490 signal to additional ligand or partial removal of ligand in the differentiated PC12 cells (Figures 2 and 3) could be explained
by receptor internalization after prolonged and continuous exposure to NGF. Recent studies from several different groups indicate that internalized TrkA remains activated residing in 'signaling endosomes' (28-30). To address the possibility that TrkA was internalized in the differentiated PC12 cells, we analyzed the relative level of TrkA on the cell surface of the differentiated PC12 cells. Consistent with reports demonstrating that receptor activation triggers internalization (31,32), cell surface biotinylation experiments showed that TrkA rapidly leaves the surface of naive PC12 cells upon NGF stimulation. After 7 days of continual NGF treatment, we could not detect TrkA among the streptavidin precipitated cell surface proteins (Figure 4A, lower panel). The level of total TrkA levels in cell lysates remained relatively unchanged over the 7 day course of NGF treatment (Figure 4A, upper panel). The cell surface levels of activated TrkA decreased with similar kinetics to that of cell surface TrkA (Figure 4A, lower panel). This indicates that the vast majority of the TrkA receptors, regardless of activation state, are intracellular after long term NGF treatment. Since the lysates from the differentiated cells showed a clear Trk<sup>PY490</sup> signal (Figure 4A, upper panel, lane 6), a fraction of internalized TrkA receptors remains persistently activated in the 7 day differentiated cells. Supplementing the differentiated PC12 cell cultures with an additional 50 ng/ml of NGF for 5 min did not cause any discernable change in either the cell surface or total Trk<sup>PY490</sup> levels (Figure 4B, lanes 1 and 2). Taken together, these data argue that ligand-induced receptor internalization in differentiated PC12 cells underlies the relative insensitivity of TrkA to varying NGF levels.
Persistent phosphatase activity restricts the activity of cell surface TrkA in differentiated PC12 cells

Although the biotinylation experiments suggest that very few TrkA receptors reside along the cell surface in the differentiated PC12 cells, the attenuation of phospho-TrkA levels seen after treating the differentiated cells with 1:500 anti-NGF antibody argues that a least a population of the receptors must recycle to the cell surface within a 2 h period (see Figure 3B). Treating differentiated PC12 cells with a cell permeable tyrosine phosphatase inhibitor (orthovanadate) for 2 h caused a dramatic increase in total $\text{Trk}^{\text{PY490}}$ levels with only a slight increase in cell surface $\text{Trk}^{\text{PY490}}$ (Figure 4B). This is consistent with the vast majority of both the phosphorylated and non-phosphorylated forms of the TrkA receptor residing in the intracellular pool. In keeping with this result, addition of 50 ng/ml NGF caused no detectable change in total or cell surface $\text{Trk}^{\text{PY490}}$ levels (Figure 4B). However, a strong $\text{Trk}^{\text{PY490}}$ signal was detected among the cell surface proteins upon shorter duration of orthovanadate treatment. At 30 min after addition of orthovanadate to the 7 day NGF-differentiated PC12 cells, the biotinylated cell surface protein preparations showed an increase in $\text{Trk}^{\text{PY490}}$ (Figure 4C, lane 2, lower panel). This fell sharply thereafter with essentially no signal detected by 4 h after orthovanadate treatment. During this same interval, the levels of $\text{Trk}^{\text{PY490}}$ in the whole cell lysates progressively increased from 30 min to 4 h with no change in total TrkA levels (Figure 4C). This indicates that persistent tyrosine phosphatase activity in the differentiated PC12 cells maintains the cell surface TrkA receptors in an inactivated state. Incubating the differentiated cells with 1:500 anti-NGF antibody prior to a 30 min orthovanadate treatment attenuated the increase in cell
surface Trk^PY490 levels (Figure 4C, lane 5). Thus, cell surface TrkA is rapidly activated by extracellular NGF when phosphatase activity is inhibited and then, the activated receptor is moved into intra-cellular pool and is inaccessible to extracellular ligand.

**Persistent TrkA activation in the differentiated PC12 cells regulates Erk1/2 and Akt phosphorylation**

In naive PC12 cells, NGF activates Erk 1/2 and Akt through signal transduction cascades that are initiated by tyrosine phosphorylation of TrkA (33,34). Since Erk 1/2 and Akt activity are important for the differentiation and survival of PC12 cells and primary neurons (2,9,35,36), we asked whether the persistent TrkA kinase activity in 7 day NGF-differentiated PC12 cells could maintain a basal level of Erk 1/2 and Akt activity. Similar to the Trk^PY490 levels, we could detect both phospho-Erk 1/2 and phospho-Akt signals in 7 day NGF-differentiated PC12 cells (Figure 5A, lane 1). Treatment with 200 nM K252a substantially decreased Erk 1/2^{PT183/PY185} and Akt^{PS473} signals within 2 h (Figure 5A, lane 2). Treating the differentiated cells with PD98059, an inhibitor of the MAP kinase kinase upstream of Erk 1/2 (37), rapidly decreased levels of Erk 1/2^{PT183/PY185} but did not affect Akt^{PS473} or Trk^PY490 levels (Figure 5A, lane 3). Conversely, treating the differentiated cells with the PI3K inhibitor, LY294002, decreased Akt^{PS473} levels without affecting Erk 1/2^{PT183/PY185} or Trk^PY490 levels (Figure 5A, lane 4). Thus, the Erk 1/2 and Akt activities in the NGF-differentiated PC12 cells are specifically regulated by TrkA. Moreover, treating the differentiated PC12 cells with 1:500 anti-NGF antibody similarly decreased Erk 1/2^{PT183/PY185} and Akt^{PS473} levels indicating that basal Erk 1/2 and Akt activities in differentiated PC12 cells are NGF-
dependent (Figure 3B). Basal activity of TrkA and PI3K was required for survival of the PC12 cells in these experiments, since both K252a and LY294002 treatment significantly reduced survival of the 7 day differentiated PC12 cells (Figure 5B). The depletion of Erk 1/2 activity by PD98059 had no significant effect on survival of the differentiated PC12 cells in these studies (Figure 5B).

**Basal TrkA activity maintains ongoing transcription in differentiated PC12 cells**

Neuronal differentiation of PC12 cells requires new gene expression (8). Once fully differentiated, the PC12 cells require continual transcription to mount a rapid regenerative response to neurite injury (38). Thus, it is likely that the basal activity of TrkA and downstream signaling pathways in the differentiated PC12 cells maintains NGF-dependent transcription in the neuronal PC12 cells. During the initial stages of NGF-induced PC12 cell differentiation, immediate early gene transcription is activated at least in part through TrkA-dependent activation of Erk 1/2 (39). c-jun and c-fos family members, CREB, and Elk1 and serum response factor [SRF] regulate gene expression by binding to their respective enhancer elements AP1, CRE, and SRE, respectively (40-42). These transcription factors are regulated by post-translational modifications, including phosphorylation by Erk 1/2 or protein kinases directly or indirectly regulated by Erk 1/2 (43-45). The persistent Erk 1/2 activity that we detected in the differentiated PC12 cells above raises the possibility that AP1-, SRE-, and CRE-dependent transcription could also be maintained by NGF in the differentiated PC12 cells. In the experiments below, we used PC12 cells stably transfected with AP1, SRE and CRE reporter constructs driven by simple promoter elements to explore this possibility.
The stably transfected cell lines used to examine the transcriptional responses in differentiated PC12 cells showed rapid induction of luciferase activity in response to PMA in the AP1-Luc and SRE-Luc lines (Figure 6A) and dbcAMP in the CRE-Luc lines (see Figure 7). The naive AP1-Luc and SRE-Luc PC12 cells exhibited an approximate 4 fold and 8 fold induction, respectively, in luciferase activity by 5 h after NGF exposure (Figure 6A). Pretreatment with 200 nM K252A prevented the NGF-dependent increase in luciferase activity in the AP1-Luc and SRE-Luc cell lines indicating that NGF activation of TrkA regulated this increase in luciferase expression (Figure 6A). We consistently detected a basal of luciferase activity in the 7 day differentiated AP1-Luc and SRE-Luc cell lines. This luciferase activity depended on NGF maintenance of TrkA activity since treatment of the differentiated AP1-Luc and SRE-Luc cell lines with 200 nM K252a caused a fall in luciferase activity within 20 h (Figure 6B). Treating the 7 day differentiated AP1-Luc and SRE-Luc cell lines with 1:500 anti-NGF antibody for 20 h caused a fall in luciferase activity comparable to K252a treatments (Figure 6B). Thus, NGF-dependent activation of TrkA both initially activates and maintains AP1- and SRE-dependent transcription in PC12 cells.

The initial activation of luciferase transcription in the CRE-Luc cell lines was quite different from that seen in the AP1-Luc and SRE-Luc cell lines. CRE-dependent transcription only increased 2-2.5 fold at 5 h after NGF treatment compared to luciferase levels in unstimulated CRE-Luc lines (Figure 7). Over the same time course, dbcAMP increased luciferase activity by more than 13 fold above unstimulated levels in the CRE-Luc lines (Figure 7). In contrast to AP1 and SRE-dependent transcription where K252a near completely blocked the effect of NGF, pretreating the CRE-Luc lines with K252a
did not affect the modest change in luciferase activity that NGF appeared to activate (Figure 7). Considering that NGF rapidly activates CREB by inducing phosphorylation on serine 133 (46), it is surprising that NGF did not cause a large increase in CRE-dependent transcription in the naive PC12 cells. After 7 day of NGF treatment, the CRE-Luc cell lines showed greater than 25 fold more luciferase activity than non-treated cells (Figure 7). Importantly, the increased luciferase activity in the 7 day differentiated CRE-Luc lines was decreased by 20 h treatment with K252a (Figure 7). Treatment with 1:500 anti-NGF antibody similarly decreased the CRE-dependent luciferase activity in the differentiated CRE-Luc cell lines. Thus, the CRE reporter construct used here is not initially responsive to TrkA signaling but transitions to an NGF-responsive state as the PC12 cells differentiate.
DISCUSSION

PC12 cells have long been used as an *in vitro* model of neuronal differentiation. Over the course of 3-7 days NGF treatment, the PC12 cells establish a sympathetic neuron-like phenotype with a network of neurites (15). This change in phenotype requires new gene expression (8). Considering the natural turnover of cellular proteins, maintaining this newly differentiated phenotype undoubtedly requires continual transcription of mRNAs encoding proteins needed for structure and function in the neuronal PC12 cells. Indeed, rapid regeneration of sheared neurites from differentiated PC12 cells is regulated by translation of short-lived mRNAs indicating that continual transcription is needed to maintain this injury response (38). Differentiated PC12 cells also require continual exposure to NGF to maintain their survival (47). Despite the need for continual exposure to NGF, most kinetic analyses of NGF signal transduction have shown that activities of its TrkA receptor and downstream protein kinases rapidly peak and then fall to seemingly undetectable levels within hours after ligand stimulation (11,23,34,48). This rapid peak and fall in NGF signal transduction is not consistent with the notion that continual NGF exposure is needed to support neuronal differentiation and survival in the PC12 cells. Data from Zhou *et al.* (1995) suggested that prolonged activation of TrkA is needed to drive neuronal differentiation of PC12 cells (23). Similarly studies by Xia *et al.* (1995) indicated that NGF maintains survival of the differentiated PC12 cells by regulating activity of Erk 1/2 (9). Here we show that an activating phosphorylation of TrkA on tyrosine 490, the SHC-binding site (49,50), is maintained in the PC12 cells over the entire course of neuronal differentiation (Figure 2A). This basal activity of TrkA in the differentiated PC12 cells is NGF-
dependent and maintains activity of the Ras/Rap\textsuperscript{Æ} Erk and PI3K\textsuperscript{Æ} Akt signaling pathways (Figure 4A). Pharmacological inhibition of TrkA with K252a or of PI3K with LY294002 results in death of the differentiated PC12 cells indicating that the basal levels of activity of these molecules in neuronal PC12 cells are biologically relevant for NGF-dependent survival (Figure 4B). The sustained TrkA activity is also biologically relevant for maintaining gene expression since removal of NGF or inhibition of TrkA resulted in decreased transcription from simple CRE, SRE, and AP1 promoter elements in the 7 day differentiated PC12 cells.

The magnitude of Trk\textsuperscript{PY490} in the 2-7 day differentiated PC12 cells is approximately 15 fold less than the peak in Trk\textsuperscript{PY490} levels seen at 5 min after initial stimulation of PC12 cells. This is similar to the level of phospho-TrkA that Zhou et al (1995) observed in TrkA immunoprecipitates from 7 day differentiated PC12 cells detected using anti-phosphotyrosine antibodies (23). It was surprising how stable the Trk\textsuperscript{PY490} signal appeared in 7 day differentiated PC12 cells despite significantly altering the NGF concentration by addition of two fold more ligand or partial removal of ligand using neutralizing anti-NGF antibodies (Figures 2A and 3B). Sequestration of TrkA receptors away from extracellular sources of ligand or continual inhibition of the TrkA receptor activity at the cell surface could explain these findings. TrkA is internalized after it binds to ligand (51-53). 'Signaling endosomes' have been isolated from PC12 cells and likely occur in primary neurons (54-56). Such endosomes provide sequestered TrkA that is biologically active, yet not accessible to extracellular ligands. The half-life of TrkA along the surface of PC12 cells decreases remarkably after NGF treatment indicating that ligand binding initiates more rapid receptor internalization (57).
Our studies are consistent with rapid internalization of TrkA after NGF binding but further indicate that both active and inactive receptors remain internalized and inaccessible to extracellular NGF in the differentiated PC12 cells. Previous studies have shown that the fall in phospho-TrkA levels that follows peak TrkA activation in naive PC12 cells can be attributed to receptor dephosphorylation by phosphatases (58,59). The data presented in Figure 3 suggest that TrkA activity is similarly downregulated by phosphatases over the course of neuronal differentiation. Since high-affinity NGF binding sites can be detected on the surface of differentiated PC12 cells by $^{125}$I-NGF binding assays (23), a population of TrkA receptors must remain accessible to extracellular NGF in the differentiated PC12 cells. The attenuation of Trk$^{PY490}$ levels that we saw after treating the differentiated cells with neutralizing concentrations of anti-NGF antibody suggests that TrkA receptors recycle to the cell surface over a 2 h period (Figure 2B). The rapid appearance of phospho-TrkA among the cell surface proteins after orthovanadate treatment of the differentiated PC12 cells argues that continual tyrosine phosphatase activity maintains cell surface TrkA in a relatively inactive state despite the presence of saturating ligand concentrations. Rapid internalization of TrkA after its activation by NGF provides a further mechanism to restrict TrkA from extracellular ligand changes by intracellular sequestration. Thus, the cell surface TrkA receptors are maintained in a refractory state by ongoing dephosphorylation in the differentiated PC12 cells. Whether the relevant phosphatase is constitutively active or NGF-stimulated remains to be determined. However, the observation that brief removal of NGF from differentiated PC12 cells restores the NGF stimulation of TrkA to a level
comparable to naive cells (23) suggests that the phosphatase activity may also be NGF responsive.

The NGF-dependent activity of TrkA in the differentiated PC12 cells also maintained a basal level of Erk 1/2 and Akt activation. Treatment with the MEK1 inhibitor, PD98059, argues that activation of Erk 1/2 by NGF in the differentiated PC12 cells is MEK1-dependent as has been demonstrated in naive PC12 cells (60). Similarly, studies with the PI3K inhibitor, LY294002, indicate that NGF maintains activity of Akt in the differentiated PC12 cells through the PI3K pathway just as it does during the initial activation of Akt by NGF (61). Pharmacological inhibition of TrkA or PI3K, but not MEK1, decreased survival in the differentiated PC12 cells (Figure 5B). This finding is consistent with previous studies that TrkA → PI3K → Akt signaling cascade is needed for serum-free survival in the differentiated PC12 cells (2). Since inhibition of MEK1 in the differentiated PC12 cells did not affect their survival, our findings argue against any substantial role for Erk 1/2 in NGF-dependent survival of the differentiated PC12 cells.

In contrast to our findings, Xia et al. (1995) reported that NGF-dependent survival in PC12 cells is determined by a balance between activities of Erk 1/2 and the stress-activated protein kinases, JNK and p38MAPK (9). However, Xia and colleagues reached this conclusion by inhibition or activation of JNK and p38MAPK pathways rather than directly inhibiting the Ras/Rap → Erk 1/2 pathway as we have done here. Crosstalk between constituents of the PI3K → Akt pathway and stress-activated protein kinase could account for differences between our findings. The effects of Akt appear more directly linked to neuronal survival than do those of Erk 1/2. It is intriguing that Akt levels appeared to remain for longer periods in the differentiated PC12 cells after
treatment with K252a or anti-NGF antibodies compared to levels of Erk 1/2$^{PT183/PY185}$ (Figure 3B). This suggests that the PI3K–Akt pathway is more resistant to NGF withdrawal from the differentiated PC12 cells than is Ras/Rap–Erk 1/2 pathway. Such would argue that neuronal wellness or health is compromised before survival is affected in the differentiated PC12 cells. After NGF withdrawal from developing sympathetic neurons, neurite retraction is seen before the neurons commit to apoptosis. Consistent with our findings in the PC12 cells, NGF-dependent survival of the sympathetic neurons appeared dependent on PI3K activity rather than Erk 1/2 activity (35).

Stable transfection of the PC12 cells provided us with a means to address how NGF maintains transcription after the PC12 cells have initiated their neuronal differentiation. Both activation and maintenance of AP1- and SRE-dependent transcription were regulated by NGF-dependent TrkA activity (Figure 6). Thus, both the initial burst of TrkA activity in the naive cells and the sustained TrkA activity in the differentiated cells contribute to NGF's regulation of transcription. Considering half-life of the luciferase mRNA and protein, the fall in SRE- and AP1-dependent transcription in the differentiated PC12 cells must have occurred well before we were able to detect decreased luciferase activity. Thus, loss of transcriptional activity, at least from these simple promoter elements, occurs prior to when survival of the PC12 cells is significantly compromised.

The magnitude of SRE- and AP1-dependent transcription was comparable to the level of TrkA activity in both the naive and differentiated PC12 cells, but CRE-dependent transcription did not show such a clear relationship to TrkA activity. In the naive state, CRE-dependent luciferase activity was only minimally increased by NGF and this was
not inhibited by the K252a over the first 24 h of NGF exposure (Figure 6). Minneman et al. (2001) recently reported a similar lack of NGF-responsiveness of a simple CRE promoter construct in naive PC12 cells (62). Similarly, deletion analysis of the c-fos promoter indicated that the CRE alone is not sufficient to activate transcription after NGF stimulation in naive PC12 cells indicating that CREB likely requires additional transcriptional regulators (16). Neither of these studies considered CRE-dependent transcription after longer durations of NGF treatment in the PC12 cells. The stable transfectants allowed us to assess transcription in both the naive and differentiated state using the same cell line. CRE driven luciferase expression became TrkA-dependent in the differentiated state. Thus, TrkA specific induction of CRE-dependent transcription is delayed beyond the initial burst in TrkA protein kinase activity despite that this TrkA activation causes a rapid activation of CREB (11,45). The minor increase in CRE-dependent transcription seen after NGF stimulation of the naive PC12 cells could be attributable to the low affinity neurotrophin receptor, p75LNTR. NGF or BDNF can stimulate calcium uptake through the p75LNTR in non-Trk receptor expressing cells (63) and increased intracellular calcium can stimulate CRE-dependent reporter activity in a TrkA-independent manner (64,65).

Although serine 133 phosphorylation results in activation of CREB, several reports have shown that this CREB phosphorylation is not always sufficient for induction of CRE-dependent gene expression (16,66,67). Recruitment of the CREB binding protein, CBP, is sufficient for CREB-mediated gene activation (68). CBP binds to components of the basal transcriptional machinery and may act as a scaffold to stabilize the preinitiation complex at the promoter (69). Recruitment of CBP to CREB via
phosphorylated serine 133 has been shown to be sufficient for inducing transcription in several different experimental systems (70,71). However, absence of CBP in the naive PC12 cells cannot explain the transition to NGF responsiveness that we saw in CRE-dependent transcription since cAMP caused a large increase in luciferase activity in the naive CRE-Luc lines (Figure 7). Delayed recruitment of CBP to CREB$_{PS133}$ after NGF treatment may underlie the transition of CRE responsiveness to NGF-dependent Trk activity. CBP also has intrinsic histone acetyltransferase activity (72), suggesting that it may also play an important role in chromatin remodeling. Alterations of chromatin structure could effectively regulate how other transcriptional machinery binds to the CRE promoter that we have used. Recent studies in Aplysia indicate that such histone acetylation determines whether serotonin can activate CRE-dependent transcription in sensory neurons (73). Additional studies will be required to determine whether CRE in PC12 cells is subjected to such epigenetic regulation as it acquires TrkA-dependence.
REFERENCES


FIGURE LEGENDS

Figure 1: Activation kinetics of TrkA, Erk 1/2, and Akt in NGF stimulated PC12 cells

Serum-starved PC12 cells were stimulated with 50 ng/ml NGF for 0 to 15 h. Lysates were normalized for protein content and used for immunoblotting with phosphospecific antibodies specific for Trk$^{PY490}$, Erk 1/2$^{PY183/PT185}$, and Akt$^{PS473}$.

A, In response to NGF, Trk$^{PY490}$ levels peaked at approximately 5 min (lane 2) and declined to undetectable levels by 12 h (lane 8).

B, Similarly, Erk 1/2$^{PY183/PT185}$ levels peaked at 5 min (lane 2) and declined to undetectable levels by 12 h (lane 8).

C, Akt$^{PS473}$ levels peaked by 5 min (lane 2), remained maximally active for up to 1 h (lane 5), and then progressively declined.

D, Reprobing the same blots with a polyclonal antibody specific for nonphosphorylated Erk 1/2 showed similar Erk 1/2 protein levels in all samples. Similarly, levels of nonphosphorylated TrkA and Akt were relatively equivalent in these lanes [data not shown].
Figure 2: TrkA remains phosphorylated in PC12 cells over the course of NGF differentiation

PC12 cells were stimulated with 50 ng/ml NGF in serum-free conditions for the indicated periods. An additional 50 ng/ml NGF or 200 nM K252a were added to 7 day NGF stimulated cultures 24 h after the media was last replaced.

A, By extending exposure time and using optimized detection systems following Western blot analysis of cell lysates with anti-Trk<sup>PY490</sup> antibodies as described in Figure 1, a basal level of Trk<sup>PY490</sup> was detected 48 h after initial NGF exposure of naive PC12 cells (lane 6). This persistent Trk<sup>PY490</sup> signal was also detected in 7 day NGF stimulated PC12 cells (lane 7). Albeit low, this basal phospho-TrkA level is easily distinguished from the anti-Trk<sup>PY490</sup> signals in the unstimulated cells (lanes 5-7 vs. lane 1). Exposure of 7 day NGF-stimulated PC12 cells to an additional 50 ng/ml NGF for 5 min did not cause any detectable increase in Trk<sup>PY490</sup> levels (lanes 7 and 8).

B, To compare the levels of Trk<sup>PY490</sup> present in 7 day NGF-stimulated PC12 cells to the peak TrkA phosphorylation initially activated by NGF in naive PC12 cells, varying amounts of lysates from NGF-treated naive PC12 cells (50 ng/ml NGF, 5 min) were compared to lysates from 7 day NGF-stimulated PC12 cells. This titration was repeated with different amounts of lysates from naïve PC12 cells (50 ng/ml NGF, 5 min) to obtain a tighter correlation between the magnitudes of phospho-TrkA levels in naïve vs. differentiated PC12 cells [data not shown]. 200 nM K252a caused a fall in Trk<sup>PY490</sup> levels, indicating that the signal represented phosphorylated TrkA (lane...
9). Trk\textsuperscript{PY490} levels were evaluated by immunoblotting with an anti-phospho Trk antibody as described above.
**Figure 3:** Persistent phosphorylation of TrkA in differentiated PC12 cells is NGF-dependent

**A,** To determine the concentration of anti-NGF antibody (Sigma) required to fully and partially block NGF-induced TrkA activation, the antibody was titrated in naive PC12 cells. Dilutions of anti-NGF antibody were preincubated with 50 ng/ml NGF in serum-free media for 20 min and then this mixture was added to serum-starved PC12 cells for 5 min. Trk\(^{\text{PY490}}\) levels were evaluated by immunoblotting with anti-Trk\(^{\text{PY490}}\) antibodies as described above. 1:500 antibody dilution completely blocked TrkA activation (lane 1) while 1:2500 antibody dilution decreased TrkA phosphorylation to levels comparable to a 5 ng/ml NGF treatment (lane 6, top panel vs. lane 4, bottom panel).

**B,** PC12 cells were stimulated with 50 ng/ml NGF for 7 days as described above and then treated directly with anti-NGF antibody for the times indicated. 1:500 anti-NGF antibody treatment caused a fall in Trk\(^{\text{PY490}}\), Erk 1/2\(^{\text{PT183/PY185}}\), and Akt\(^{\text{PS473}}\) levels by 2 h (lane 3), which further declined to undetectable levels by 24 h (lane 6). Trk\(^{\text{PY490}}\), Erk 1/2\(^{\text{PT183/PY185}}\), and Akt\(^{\text{PS473}}\) levels were not significantly changed by a 1:2500 anti-NGF antibody treatment even after 24 h (lane 6) despite that the same dilution decreased initial activation of TrkA to levels comparable to stimulation with 5 ng/ml NGF (Figure 3A, lane 4). Probing blots with an antibody to total TrkA indicated equal levels of TrkA protein in all samples.

**Figure 4:** Responsiveness of TrkA to extracellular NGF is restricted in differentiated PC12 cells.
Cell surface Trk\(^{PY490}\) and TrkA protein levels were determined in naive vs. 7 day NGF-differentiated PC12 cells by cell surface biotinylation (see “Materials and Methods” for details) followed by streptavidin precipitation. The upper series of images show immunoblotting of total protein and the lower series show immunoblotting of cell surface protein isolated by streptavidin precipitation. Immunoblotting with anti-Trk\(^{PY490}\) or anti-TrkA antibodies was performed as described above. Biotinylation of only cell surface proteins was confirmed by reprobing blots with an antibody to eIF5. No eIF5 was detected in the cell surface protein isolation.

A, Naive PC12 cells were stimulated with 50 ng/ml NGF for the indicated times. Consistent with the kinetics of TrkA activation shown in Figure 2A, the Trk\(^{PY490}\) signal from total cell protein exhibited rapid peak activation and a subsequent fall to basal levels within hours after NGF stimulation, despite relatively no change in total TrkA levels during exposure to NGF for 7 days (upper panel). The cell surface TrkA was also rapidly phosphorylated after NGF treatment (lower panel). Cell surface TrkA levels continued to decline with longer NGF exposure, and fell to undetectable levels by 7 days (lane 6). Streptavidin precipitation of non-biotinylated lysates from naive PC12 cells treated with NGF for 5 min show specificity of the precipitates (no biotin; lane 1).

B, PC12 cells were stimulated with 50 ng/ml NGF for 7 days and then treated with 1 mM sodium orthovanadate (Na\(_3\)VO\(_4\)) for 2 h (lanes 3 and 4). Basal Trk\(^{PY490}\) signal detected in 7 day NGF-differentiated PC12 cells dramatically increased upon Na\(_3\)VO\(_4\) treatment (lane 1 vs.3, upper panel). Supplementing Na\(_3\)VO\(_4\)-treated differentiated PC12 cell cultures with an additional 50 ng/ml of NGF for 5 min did not
discernibly alter the TrkPY490 signal (lanes 3 vs. 4, upper panel). In contrast to the
total TrkA, Na3VO4 treatment caused only a slight change in TrkPY490 levels at the
cell surface (lane 3, lower panel).

C, PC12 cells were stimulated with 50 ng/ml NGF for 7 days and then treated with 1 mM Na3VO4 for the indicated times. Total cellular TrkPY490 signal steadily increased following Na3VO4 treatment (lanes 2-4, upper panel). However, this increase was attenuated when the cultures were pretreated with 1:500 anti-NGF antibodies for 4 h prior to Na3VO4 (lane 5, upper panel). In contrast to total TrkA, cell surface TrkA became phosphorylated by 30 min of Na3VO4 treatment, but quickly diminished with longer treatment (lanes 2-4, lower panel). The anti-NGF treatment prior to Na3VO4 blocked cell surface TrkA activation by Na3VO4 (lane 5, lower panel).

**Figure 5:** Basal TrkA activity regulates Erk and Akt activation status in
differentiated PC12 cells

A, PC12 cells were stimulated with 50 ng/ml NGF for 7 day and then treated with
200 nM K252a, 50 μM PD98059, or 50 μM LY294002 for 2 h. Erk 1/2 and Akt phosphorlyation were evaluated by immunoblotting with phosphospecific-antibodies specific for Erk1/2PT183/PY185 and AktPS473, respectively. K252a treatment decreased Erk1/2PT183/PY185 and AktPS473 signals to undetectable levels by 2 h. PD98059 treatment decreased Erk1/2PT183/PY185 (lane 3) and LY294002 treatment decreased AktPS473 (lane 4) to levels that were similar to K252a treatment. Nonphosphorylated Erk 1/2 levels were similar in DMSO and drug treated cells indicating equal loading of protein in both samples.
B, PC12 cells were treated with NGF for 7 day and then with K252a, PD98059 or LY294002 as above. The percentage of surviving cells was determined by MTT assay performed at 24 and 48 h after addition of inhibitors. Results are displayed as percentage of signal in sister cultures that had been treated with equivalent volume of DMSO (vehicle control) for identical duration. Experiments were performed in triplicate and are reported ± standard deviation.

Figure 6: Persistent TrkA activity in differentiated PC12 cells maintains AP1-, SRE-, and CRE-dependent transcription

A, Stable cell lines carrying the luciferase reporter gene regulated by the AP1 (AP1-Luc) or SRE (SRE-Luc) promoter elements were stimulated for 5 h with 1 µM PMA (hatched columns) or 50 ng/ml NGF (white columns). Pretreatment with 200 nM K252a (dark grey columns) for 30 min prior to NGF treatment blocked AP1 and SRE reporter induction. Luciferase units (relative light units [RLU]) were normalized to protein content and are presented as fold induction over unstimulated cells. Error bars represent standard deviation of triplicate samples.

B, Stable PC12 cells lines were stimulated with 50 ng/ml NGF for 7 day and then treated with 200 nM K252a or 1:500 dilution of anti-NGF antibody for 20 h (white columns = NGF; dark grey columns = NGF + K252a; light grey columns = NGF + anti-NGF antibody). 200 nM K252a decreased AP1 and SRE reporter activity by more than 75 % relative to DMSO treated NGF-differentiated cells. 1:500 dilutions of anti-NGF antibody caused similar falls in AP1 and SRE reporter activity, respectively. Relative light units [RLU] were normalized to protein content and are
presented relative to 7 day NGF-treated treated with equal volumes of vehicle (DMSO). Error bars represent standard deviation of triplicate samples. Representative data from three independent experiments are shown. All experiments were performed in triplicate in at least 2 stable clones for each reporter.

Figure 7: CRE-dependent transcription undergoes a transition in NGF-responsiveness during neuronal differentiation

Relative reporter activities of the stable CRE-Luc PC12 cell lines after exposure to 1 µM dbcAMP for 5 h (hatched column) or NGF for 5 h and 7 days are shown (white columns). RLU was normalized for protein content and is presented relative to levels in unstimulated CRE-Luc lines. Compared to the dbcAMP response, the naive cells show little change in luciferase activity in response to NGF and this was not affected by pretreatment with 200 nM K252a (dark grey columns). In contrast, the 7 day differentiated cells show luciferase activities more than 25 fold above the levels in unstimulated cells and this was attenuated by treatment with 200 nM K252a (dark grey column) or 1:500 dilution anti-NGF antibody (light grey column) for 20 h prior to harvest. Error bars represent standard deviation of triplicate samples. Representative data from three independent experimental repetitions are shown that were performed on multiple CRE-Luc clones.
Figure 1
JH Chang, EA Mellon, NC Schanen, JL Twiss
Figure 2

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Figure 3

A

anti-NGF

NGF (ng/ml) 0 0.5 1 5 10 15 25 50

Trk

Trk

B

anti-NGF

2 h 24 h

Trk

TrkA

Akt

Erk 1/2

Erk 1/2
Figure 4
JH Chang, EA Mellon, NC Schanen, JL Twiss
Figure 5
JH Chang, EA Mellon, NC Schanen, JL Twiss

A

![Western Blot Image](image)

B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h</th>
<th>48 h</th>
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<tbody>
<tr>
<td>K252a (200 nM)</td>
<td>64.2 ± 5</td>
<td>18.9 ± 12</td>
</tr>
<tr>
<td>PD98059 (50μM)</td>
<td>103.7 ± 16</td>
<td>94.6 ± 7</td>
</tr>
<tr>
<td>LY294002 (50μM)</td>
<td>75.5 ± 19</td>
<td>24.4 ± 8</td>
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Figure 6
JH Chang, EA Mellon, NC Schanen, JL Twiss
Persistent TrkA activity is necessary to maintain transcription in neuronally differentiated PC12 cells
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