Cyclic-AMP Response Element-Based Signaling Assays for Characterization of Trk Family Tyrosine Kinases Modulators

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Abstract

Neurotrophins (NTs) induce gene transcription by binding their high-affinity tropomyosin-related kinase (Trk) receptors and initiating intracellular signal transduction cascades. In particular, activation of the cyclic AMP response element (CRE) in the promoters of target genes serves as surrogate markers for Trk receptor activation as demonstrated in both in vivo and in vitro systems. We used a HEK293 cell line stably expressing a CRE-luciferase reporter gene to develop an assay for monitoring Trk activation in response to their cognate ligands. Using TrkB, we showed that the assay was sensitive to physiological concentrations of brain-derived neurotrophic factor (BDNF) and that the signal was sufficiently robust to be suitable for implementation in high-throughput format. Further characterization of the TrkB expressing stable cell lines showed high-affinity binding for BDNF, a high density of receptor expression, and supported BDNF-mediated phosphorylation signaling. Consistent with this, inhibitors of phosphatidylinositol 3-kinase and the phospholipase C- γ pathways led to reduction of BDNF-mediated luciferase responses. In contrast, inhibitors of mitogen-activated protein kinase pathways further potentiated BDNF responses.

This assay was NT-Trk receptor pair-selective and shown to be further applicable to other Trk family members. This assay may be useful in screening compound libraries to identify Trk agonists, which may be applied towards discriminating between the activities of the different Trk receptor family members and the development of pharmacological drugs.

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Introduction

Neurotrophins (NTs) are large secreted peptides important for the development of the nervous system and for maintaining neuronal integrity and plasticity through adulthood. NTs constitute a family of four members that bind a set of multi-domain single transmembrane receptors: the low-affinity glycoprotein p75 receptor and a family of high-affinity tropomyosin receptor kinases (Trk) [1]. The p75 receptor binds all the NTs, and its physiological role is most clearly understood for its association with processes that lead to cell death and apoptosis [2]. The Trk receptors, in contrast, are selective for the different NTs and mediate cell survival processes [3]. The cognate NTs for the Trk receptors are nerve growth factor (NGF) for TrkA, brain-derived neurotrophic factor (BDNF) and NT4/5 for TrkB and NT3 for TrkC, although the latter also has a low affinity for TrkA and TrkB [4, 5].

NTs bind and relay biological functions through homodimers or heterodimers of p75 and Trk receptors. The multiplicity of the NT system and their validated roles in neurodegenerative diseases has inspired considerable interest in identifying small non-peptide NT mimetics. Such compounds may provide reagents to probe how the different receptors influence cell fate and higher order functions, as well as drugs for pharmacological intervention.

NTs are peptides of approximately 120 amino acids that share about 50% homology. The active NT moiety is a pseudosymmetrical dimer in which the β -sheets of each monomer align to form a central core and the loops remain exposed [6]. On the other hand, the Trk receptors are multi-domain transmembrane proteins with approximately 800 amino acids. It has been shown that the ligand binding is promoted by two sets of contacts on the last domain of TrkB (D5/Ig2) [7]. One contact region is between the β -sheet of NT and loops at the C-terminal pole of Trk-D5/Ig2, and the second is between the N-terminal residue of NT and the face of one β -sheet of the Trk-D5/Ig2. The first interaction involves 5 of the 9 residues of a domain, which is dubbed the 'conserved' patch for its high sequence homology among the NT. The second is with 3 of the 7 residues in a region that is more dissimilar among the NT, hence referred to as the 'specificity patch'. Specificity and high-affinity interactions between Trk receptors and NT ligands are also to be mediated by the loop regions of NTs, as shown by site-directed mutagenesis studies [8]. The paradigm where a few specific and spatially restricted residues are sufficient to generate a liganded receptor has spurred considerable effort in identifying Trk-specific agonists, leading to discovery of small molecules and peptidomimetic ligands [6, 9, 10].

Binding of NTs to their cognate Trk receptor is believed to induce receptor dimerization and subsequent receptor protein conformational changes, which lead to the activation of intracellular tyrosine kinase domain, although pre-existing receptor oligomers have also been identified in a heterologous overexpression system [11–13]. Phosphorylation of the tyrosine kinase activator domains causes the activation of multiple phosphorylation-dependent signal transduction cascades, such as the Ras/mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K)/Akt kinase pathway and the phospholipase C- γ (PLC) pathway [1, 14–16]. Several of these signal transduction cascades converge on transcriptional regulation of target genes by virtue of modulating the activity of a common transcription factor. One well-characterized enhancer is the cAMP response element (CRE), which is considered a key player in multiple

neuronal processes that promote normal mammalian physiological and psychological well-being [17]. For example, CRE-induced gene transcription is crucial for BDNF-mediated long-term synaptic plasticity and CREB activation regulates the pro-survival effects of BDNF [18, 19]. Therefore, activation of CRE is indicative of many of the therapeutic benefits of BDNF activity.

Several assays have been reported in the pursuit of identifying activators of transmembrane receptors in general and Trk receptors in particular. These may include approaches that monitor ligand-promoted dimerization/oligomerization of receptors, transcriptional activity assays using either reporter genes or endogenous genes, and biochemical assays that assess protein phosphorylation [20]. Although phosphorylation analyses are the most proximal measure of receptor activation and hence likely to identify the least non-specific hits, they require multiple steps that are incompatible with high-throughput screening, such as immunopurification of receptors and phosphorylation of an exogenous substrate [21–23]. The assays to measure receptor dimerization are based on the general thought that receptor dimerization is a key step required for downstream signaling events. They rely on two-hybrid interaction of epitope-tagged receptor constructs, such as dihydrofolate reductase [24]. However, this approach also has limitations in that dimerization may be insufficient for receptor activation, preformed dimers may exist, and/or a conformational change in a receptor monomer may be sufficient to activate the receptor. In contrast, the tractability of transcriptional activation of target genes is historically well validated in the use of a luciferase reporter gene. For example, a small non-peptidyl molecule, SB-247464, was identified for granulocyte colony-stimulating factor (G-CSF) receptors using this approach [25]. However, in the case of TrkB, a transcriptional assay with a robust signal has not been achieved in several reports, as exemplified by a 2- to 3-fold induction of luciferase gene by 100 ng/ml BDNF in PC12 cells transiently transfected with TrkB [26]. Considering that all reported NT mimetics so far were identified by a rational design, selected from limited libraries of peptides or peptidomimetics mimicking the structure of NT at key interaction sites [9, 10, 27], there is a great need for a new improved assay for discovery of small molecules with novel chemical properties [20].

In this report, we describe a development of CRE-luciferase reporter gene assay and its application towards quantitatively measuring the activities of Trk receptors. We initially focused on characterization of the TrkB system as a prototype of the NT receptors. The CRE-luciferase

ase-based TrkB assay represents a robust, sensitive, dose- and time-dependent response that was specific to BDNF. BDNF binding could be competed by supplementing the media with a soluble extracellular domain of TrkB (TrkB-Fc) or with inhibitors of signal transduction pathways. In addition, activation of TrkB was confirmed by the autophosphorylation of key intracellular residues and intermolecular phosphorylation of key effector substrates. Furthermore, the utility of the system was extended to the NGF:TrkA pair and examined for its suitability as a high-throughput screen (HTS) to aid identification of small molecular weight NT mimetics.

Materials and Methods

Transient and Stable Transfection

HEK293 cells stably expressing the 3×CRE₂-luc plasmid (Mercury Signal System, Clontech) were seeded in 10-cm² plates at a density of 7×10^5 cells/ml and the next day transfected with 5 µg of a pcDNA3.1/hygromycin (Invitrogen, Carlsbad, Calif., USA) containing a Trk isotype using Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions. The day post-transfection the cells were reseeded into 96-well white opaque plates (Nunc, Nunc) and assayed for expression of TrkB by the luciferase activity assay or divided into eight 10-cm² tissue culture plates at varying dilutions (1:20–1:80) for generation of stable cell lines. Cells plated at limiting dilutions were grown in standard media DMEM containing 10% fetal calf serum and 1% Pen/Strep, supplemented with 250 µg/ml geneticin/G418, 2 µg/ml puromycin and 350 µg/ml hygromycin until single colonies appear. Single colonies were picked by cloning cylinders, expanded, cryo-frozen for banking and screened for induction of luciferase responses following treatment with 100 ng/ml of BDNF or NGF for TrkB or TrkA, respectively. Clones with a minimum of 7-fold activities over the basal were further expanded and subject to full-dose response studies (0.8–800 ng/ml of appropriate NTs). Selected clones were maintained in their standard selection media. The parental cell line is referred to as HEK-CRE and the different stable cell lines as HEK-CRE-Trk.

Luciferase Activity Assay

HEK-CRE-Trk or HEK-CRE cells were plated at 15,000 cells/well in 100 µl growth medium in 96-well white opaque plates. The next day, the cells were treated with 10 µl of 10-fold concentrated cognate NT solution (Peprotech) and/or testing reagents as indicated in the figures. Accumulation of luciferase activities was routinely measured 16 h post-treatments, or as indicated in the figures, with Promega Steady-Glo Assay kit according to the manufacturer's protocol (Promega). In brief, media was replaced with 100 µl/well of PBS followed by 100 µl/well of Steady-Glo Assay reagent; plates were sealed with TopSeal (PerkinElmer) and shaken on a Titer Plate Shaker (Lab line instruments) at a dial speed of 5 for 5 min, and luminescence was detected in TopCount NXT v2.13 Instrument (Packard) as counts per second (CPS). Results are presented as CPS or increase over the basal activity as indicated in the figures.

Receptor Saturation Binding Assay

Saturation radioligand binding experiments using HEK-CRE-TrkB cells were conducted on both the membrane fraction and the intact cells. To prepare membranes, cells were washed with PBS, dissociated from tissue culture plates and centrifuged at 1,000 g for 10 min. The pellet was resuspended in five volumes of ice-cold 50 mM Tris, pH 7.4, with 1 mM EDTA, homogenized with a Polytron and centrifuged at 20,000 g for 20 min at 4°C; this process was repeated twice. The final membrane pellets were resuspended in a binding buffer (50 mM Tris, pH 7.4; 10 mM MgSO₄; 0.5 mM EDTA) at a concentration of 2 mg/ml and aliquots were stored at –80°C until use. Membranes were thawed on the day of the experiment and diluted to 1.5 mg/ml with the binding buffer and aliquots of 100 µl were added to each well of 96-well GF/B flat-bottom plates (PerkinElmer) containing an incubation mixture consisting of 80 µl of binding buffer and 20 µl of [¹²⁵I]BDNF (spec. act. 2,200 Ci/mmol, PerkinElmer). The non-specific binding was measured by the addition of 740 nM cold BDNF. Binding incubations proceeded at room temperature in the dark for 120 min and was terminated by filtration over a 96-well Unifilter followed by two washes with the binding buffer using Filtermate 196 Harvester (Packard). For the whole cell binding, HEK-CRE-TrkB cells were plated onto poly-D-lysine-coated 96-well tissue culture plates (BD Bioscience) at a density of 4×10^4 cells/well. After rinsing the cells 3 times with the binding buffer, aliquots of the incubation mixture were added to each well and the plates were incubated first at 37°C for 30 min and then at room temperature for another 30 min. The binding reaction was stopped by aspiration of the incubation mixture and cells were lifted using a non-enzyme dissociation buffer (GibcoBRL). Cell suspensions were transferred to 96-well GF/B plates (PerkinElmer) and filtered over a 96-well Unifilter to capture the binding complex followed by two washes. The bound complexes caught on the filter disk obtained above from both the membrane preparation and intact cells were air-dried. To each well was added 40 µl of Microscint[®]-20 scintillant and plates were heat-sealed. Plates were counted in a TopCount NXT (Packard). Specific binding was the difference between total and non-specific binding and data was analyzed by non-linear regression using Prism 4.0 (GraphPad Software Inc.).

Western Blot Analysis

HEK-CRE-TrkB stable cells were seeded in 24-well tissue culture plates at 2×10^5 cells/well in a standard medium and the next day incubated in serum-free DMEM for 90 min prior to stimulation with a cognate ligand and/or compounds as indicated in the figures. Stimulation was terminated by removal of the medium, one wash with PBS and harvesting of the cells in 100 µl of Laemmli sample buffer (Bio-Rad) preheated to 95°C. The cell suspensions were filtered through QIAshredder columns (Qiagen) and 20 µl of eluted samples were resolved on 4–12% Bis-Tris gels (Invitrogen) and processed for Western blot analysis using nitrocellulose filter membranes and semi-dry transfer apparatus (Bio-Rad). Immunoblotting was carried out using appropriate primary antibodies (anti-TrkB (1:250, Santa Cruz, sc-8613); anti-PY490 phospho-Trk (1:100, Cell Signaling); anti-MAPK (1:5,000, Cell Signaling); anti-phospho-MAPK (1:2,000, Cell Signaling)), HRP-conjugated secondary antibodies (Molecular Probes) and ECL Plus Kit (Amersham). For immunoprecipitation of phospho-TrkB, BDNF-treated HEK-CRE-TrkB cells were lysed in a stan-

dard RIPA buffer with an addition of protease inhibitors (Roche Applied Science). Two micrograms of anti-TrkB antibody (Santa Cruz, sc-8316) was added to 1 mg of lysate and the samples were incubated at 4°C overnight on a rotator. The next day, 75 µl of Protein A solution (Roche Applied Science) was added to the TrkB-antibody conjugate for additional incubation for 1 h. The resulting immunoprecipitated pellets were washed 3 times with the RIPA lysis buffer and subjected to electrophoresis on a 4–12% NuPage Tris-glycine gel and probed by Western blotting using anti-phosphotyrosine antibody G410 (0.1 µl/ml, Upstate). The signal was detected using a HRP-coupled anti-mouse secondary antibody (1:10,000, Jackson Laboratories) and the bands were visualized by ECL (Amersham) and measured using a densitometer equipped with Quantity One (Bio-Rad).

Results

Evaluation of Luciferase Responses in a Transient Transfection System

Experiments were performed to determine if BDNF treatment would lead to the accumulation of luciferase protein in HEK-CRE cells transiently expressing TrkB, according to the scheme diagrammed in figure 1. The results showed that transfection of TrkB was essential for detection of the luciferase signal in response to BDNF treatment (fig. 2a). The luciferase signal from HEK-CRE-TrkB cells, as compared to that from HEK-CRE cells transfected with vector or mock-treated, increased 10-fold over basal activity ($p < 0.001$). The assay system demonstrated a BDNF dose-dependent (fig. 2b) and time-dependent (fig. 2c) accumulation of luciferase signal. Dose-response evaluation determined the EC_{50} of BDNF to be 34.64 ng/ml (1.28 nM) (fig. 2b), which concurs with published values for physiological concentration of BDNF-mediated actions [28, 29]. These results demonstrated that the assay was a robust and sensitive functional reporter for TrkB. After BDNF treatment, a lag period of up to 4 h was followed by an increase in luciferase signal from HEK-CRE-TrkB cells, as compared to empty vector transfected HEK-CRE cells, which showed no increase (fig. 2c). From these results, 16 h treatment with BDNF was selected as a standard time for subsequent experiments.

The transient transfection approach resulted in variable transfection efficiencies that could introduce variability in measurement outcome. To circumvent this, we transfected a large batch of HEK-CRE cells and froze them in aliquots 24 h post-transfection for assays at later dates. Vials were kept frozen for a minimum of 3 days and up to 7 days, after which cells were thawed directly in 96-well assay plates to assess the effect of freeze and thaw on

Fig. 1. NT-liganded Trk monitored indirectly by transcriptional induction of a CRE-regulated luciferase reporter gene. Activation and autophosphorylation of Trk is coupled to a number of kinase cascades including Shc-MAPK, PI3K/AKT and PLC- γ , leading to the activation of CRE-regulated gene expression. HEK293 cells stably expressing a reporter cassette consisting of three CRE enhancers upstream of a promoter and luciferase reporter gene (HEK-CRE) couples activation of Trk to detection of luciferase protein accumulation, which can be detected by Promega Steady-Glo Assay kit.

transient expression of TrkB and their luciferase signal response. Cells were treated with BDNF either on the same day 6 h after the plating, or the next day approximately at 30 h, and the luciferase activity was measured after an additional incubation period of 16 h. As shown in figure 2d, at as early as 6 h post-thawing a modest increase in BDNF-induced luciferase activity was detectable in a TrkB-specific manner. The signal window was dramatically increased after a longer recovery period as demonstrated by ~7-fold change at 30 h post-thawing/replating. These results validated the assay and the use of the transient expression approach for moderate and routine throughput screening requirements.

Selection of HEK-CRE-TrkB Stable Cell Lines

Our typical high-throughput screening campaigns require cell numbers that would be better served by using stable rather than transient cell lines. We therefore made HEK-CRE-TrkB stable cell lines. Examples of the response

Fig. 2. Evaluation of the luciferase reporter activities in HEK-CRE transiently expressing TrkB. **a** Expression of TrkB selectively induces the luciferase activities. HEK-CRE cells were transiently transfected with either a TrkB plasmid or an empty vector or mock-treated. 24 h later, transfected cells were transferred to 96-well assay plates and cultured in growth media in the absence or presence of BDNF (100 ng/ml). The luciferase activities were measured following overnight incubation. **b** BDNF dose-response measured at 16 h after BDNF addition to the growth medium to HEK-CRE-TrkB cells. An EC_{50} of BDNF was determined to be 34.64 ng/ml (1.28 nM). **c** Time-points after addition of BDNF in

the HEK-CRE transfected cells when luciferase accumulations were measured. **d** Recovery of the luciferase responsiveness from cryopreservation. HEK-CRE cells in T75 flasks were transfected with either a TrkB plasmid or a vector and frozen in aliquots 24 h post-transfection in 5–6 cryovials. 3–7 days later, cells were thawed, seeded on assay plates, and BDNF was added either 6 or 30 h post-plating. 16 h post-BDNF treatment, the luciferase activity was measured as indicated in the Methods. TrkB-mediated signaling was fully recovered following freeze and thaw, resulting in time-dependent induction of luciferase activities. * $p < 0.05$ and ** $p < 0.01$, compared with TrkB alone (t test).

from 11 clones in the absence and presence of BDNF treatment are illustrated in figure 3a. Different clones showed different levels of basal luciferase activities, as well as diverse responsiveness to BDNF treatment, ranging from no to ~7-fold signal increase following BDNF treatment. Western blotting analysis (fig. 3b, and data not shown) confirmed the presence of TrkB in clones which showed BDNF-induced luciferase. We chose HEK-CRE-TrkB, clone 22, for additional characterization in this study. Clone 22 expressed a single major band of proteins recognized by a TrkB antibody that migrated at approximately

140 kDa (fig. 3b), which is consistent with the molecular weight of TrkB. Radioligand saturation binding assays with BDNF revealed an apparent dissociation constant (K_d) of 425.1 ± 168.7 and 649.5 ± 19.6 pM in cell lysates and on intact cells, respectively (fig. 3c and unpublished data, respectively), which are consistent with published values [30]. A dose response to BDNF generated an EC_{50} value of ~40 ng/ml (1.5 nM) in the reporter assay, as demonstrated in figure 3d. The potency was similar to values obtained in the transient assay (fig. 2b). In addition, these results provided compelling evidence that clone 22 medi-

Fig. 3. Characterization of HEK-CRE-TrkB stable cells. **a** HEK-CRE-TrkB stable cell lines were generated and screened for BDNF induction of luciferase responses. Results from 11 of the 39 clones tested in the absence and presence of treatment with 100 ng/ml BDNF are shown. **b** Western blot analysis with HEK-CRE-TrkB clone 22 shows a prominent band (arrowhead, TrkB) that migrated with an apparent molecular weight of approximately 140 kDa, as estimated from the molecular weight standards (arrows). **c** Saturation binding curve demonstrating specific binding of [¹²⁵I]BDNF to the membrane fraction prepared from HEK-CRE-TrkB clone 22. Binding isotherms obtained from non-linear re-

gression curve-fitting analysis are provided. Calculation of dissociation constants, K_d , from cell lysates and intact cells indicates the presence of binding sites with affinities similar to previously established ones. **d** Luciferase dose-response analysis. The luciferase response following stimulation with increasing concentrations of BDNF to HEK-CRE-TrkB clone 22 gives a sigmoidal curve with an EC_{50} of 40 ng/ml (1.5 nM). **e** Assessment of Z' factor. Luciferase activities were measured in three separate 96-well assay plates in the absence and presence of 100 ng/ml of BDNF and Z' factor [31] was calculated (Z' factor = 0.75).

Fig. 4. Luciferase responses of HEK-CRE-TrkA stable cell lines to NGF treatment. **a** 27 clonally selected HEK-CRE cells stably transfected with TrkA plasmid were screened for the responsiveness to NGF (100 ng/ml). **b** NGF dose response of HEK-CRE-TrkA clone 24. Data shown represent one of three independent experiments, which all generated similar results.

ated a BDNF response through the TrkB receptor at physiologically relevant concentrations. We further assessed the detection sensitivity of the assay using lower concentrations of BDNF and observed that 0.3 and 0.03 ng/ml BDNF led to 1.76- and 1.42-fold induction, respectively ($p = 0.0286$ and 0.001 , respectively), while 1.32-fold increase by 0.01 ng/ml BDNF was not statistically different from the basal ($p = 0.0938$). Furthermore, to determine the compatibility of clone 22 for high-capacity screening applications, the signal-to-noise ratios from multiple wells and multiple plates of HEK-CRE-TrkB cells treated in the absence and presence of BDNF were evaluated. The results are provided in figure 3e, where a calculated Z factor according to Zhang et al. [31] yielded a value of 0.75, which is within an acceptable range for HTS.

To demonstrate the versatility of this assay system, as well as to develop a counterscreen, we generated stable cell lines expressing TrkA using the same parental cell line. Figure 4 shows specific NGF-induced luciferase activities from representative 27 HEK-CRE-TrkA clones. Again, there was a close correlation between the presence of TrkA-specific band in Western blot analysis and functional luciferase response (data not shown). A dose-response to NGF with clone 24 provided an EC_{50} of 7 ng/ml (0.25 nM).

Clone 22 Stable Cells Express Functional TrkB Receptors

To confirm that the TrkB/BDNF-dependent signal transduction pathway in the neuronal system was reca-

Fig. 5. BDNF induced phosphorylation of TrkB and MAPK in HEK-CRE-TrkB stable cells. **a** Cells were incubated with the indicated concentrations of BDNF for 1 h and TrkB phosphorylation was measured by immunoprecipitation (IP) using anti-phosphotyrosine followed by Western blotting (WB) with anti-TrkB antibodies. **b** TrkB phosphorylation visualized using a phospho-Trk-specific antibody (pY490) (top blot), and compared to signal using anti- β -actin antibody (bottom blot). **c** Quantification of the dose-dependent TrkB autophosphorylation presented in **b** as a graph of ratio of the signals from matching anti-pTrk and anti-actin lanes (Y-axis) as a function of the concentration of BDNF treatment (X-axis). **d** Time-dependent TrkB phosphorylation. Cells were treated for increasing amount of time (0, 5, 10, 30, 60 min) with 100 ng/ml of BDNF and processed by WB analysis

with an anti-phospho-Trk (pY490) antibody. The band intensity of phosphoTrkB was quantified using a densitometer and graphed as a function of time. **e** WB analysis demonstrating dose-dependent inhibition of TrkB autophosphorylation by treatments with K252a, but not with K252b. **f** BDNF-mediated phosphorylation of MAPK in the presence of comparable amounts of total MAPK protein. HEK-CRE-TrkB cells were treated with 100 ng/ml of BDNF for the indicated times (top panel) or with different BDNF concentrations for 30 min (bottom panel) and processed for WB analysis with anti-phospho-MAPK antibody, anti-MAPK antibody and anti-actin antibody. Bands for immunoblotted proteins and molecular weight markers are indicated (arrows). Blots shown here are representatives of at least two independent experiments with similar results.

Fig. 6. a Induction of luciferase activities in HEK-CRE-TrkB cells is ligand-specific. HEK-CRE-TrkB clone 22 cells were treated with various trophic factors (BDNF, NGF, NT-4/5, NT-3, and bFGF) for 16 h and the resultant luciferase activities were measured. Only NT-4/5 demonstrated an EC_{50} similar to BDNF. **b** Co-treatment with TrkB-Fc prevented BDNF activation of luciferase. HEK-CRE-TrkB cells were treated with BDNF (100 ng/ml) in the

presence of increasing concentrations of TrkB-Fc and the resultant luciferase activities were measured. Addition of TrkB-Fc dose-dependently inhibited the luciferase induction mediated by BDNF. **c** TrkB-Fc protein had no effects on NGF-mediated luciferase signaling in HEK-CRE-TrkA cells, which were treated as in **b** except that NGF was substituted for BDNF. **d** Temporal window of luciferase activity in response to BDNF treatment in HEK-

pitulated in clone 22, we assessed the phosphorylation of substrates of the TrkB kinase activity using Western blot analysis. Incubation with BDNF induced dose-dependent and time-dependent increases in TrkB autophosphorylation as measured by immunoprecipitation and a direct immunoblot using a phospho-Trk-specific antibody (pY490) (fig. 5a–d). The induction of TrkB phosphorylation was suppressed by treatment with a receptor tyrosine kinase inhibitor, K252a, but not by K252b, indicating the role of a TrkB intracellular tyrosine kinase domain (fig. 5e). In addition, BDNF led to a rapid and de-novo phosphorylation of MAPK, while not affecting the total MAPK protein level (fig. 5f). Taken together, these data support the expression of functional TrkB in these cells.

Ligand Specificity and Temporal Patterns of the Luciferase Assay

In addition to BDNF, TrkB has been shown to bind NT-4/5 and NT-3. Therefore, we assessed the effects of these NTs in comparison with those of BDNF. As shown in figure 6a, the EC_{50} s of BDNF and NT-4/5 were 40 and 52 ng/ml, respectively, and the EC_{50} of NT-3 was 3-fold higher at 141 ng/ml. In contrast, induction of luciferase was undetectable in response to NGF or bFGF (fig. 6a). These results were in complete agreement with functional preference of these NT to TrkB. Neither BDNF nor NT4/5 were effective in eliciting luciferase activity in HEK-CRE parental cells ($p < 0.05$, unpublished data). To provide additional confirmation that the luciferase responses initiated by BDNF were dependent on TrkB activation, we determined if the addition of a soluble TrkB-Fc fusion protein could block the effect of BDNF. As shown in figure 6b, BDNF resulted in a 8-fold increase in the luciferase activities, which was dose-dependently inhibited by addition of TrkB-Fc protein ($IC_{50} = 0.33 \mu\text{g/ml}$, $\sim 2.5 \text{ nM}$). In contrast, TrkB-Fc protein had no effects on NGF-mediated luciferase signaling in TrkA expressing cells (fig. 6c).

The temporal window of luciferase activities was assessed at various time points after BDNF addition. Con-

sistent with transient expression, the induction of the luciferase activities was minimal up to 6 h, after which it gradually increased and the activity peaked between 16 and 22 h (fig. 6d). Accordingly, most of our assays in the study were performed at 16 h of BDNF incubation. This result showed that the signaling modulation and the luciferase readout were temporally quite stable, which should facilitate the transfer of the assay to a routine screening procedure. We also examined the effects of different concentrations of serum on the response sensitivity (fig. 6e). Comparing a normal 10% serum-containing growing condition with a 2% serum composition, there was a slight reduction in the maximum signal window detected in the cells maintained in 2% serum (12-fold increase in 2% vs. 15-fold increase in 10% serum condition). EC_{50} values, however, were not affected by either assay condition. Furthermore, good signal separation was observed even with 0.5% or no serum-containing conditions with comparable EC_{50} values (33 and 47 ng/ml for 0.5% and no serum conditions, respectively), suggesting that lower serum conditions could be used in a compound screening assay thus removing artifacts caused by serum binding as well as substantially reducing reagent costs.

Pharmacological Characterization of the TrkB Luciferase Assay

The signals of TrkB activation are known to be transduced via intracellular signaling cascades involving mitogen-activated kinase (ERK), PI3K/Akt and PLC- γ pathways [32] (fig. 1). Therefore, we assessed whether the pharmacological inhibitors of these pathways would lead to the reduction of the luciferase activity initiated by BDNF. Pretreatment with wortmannin, a PI3K inhibitor, U73122, a PLC- γ inhibitor, and thapsigargin effectively antagonized the effects of BDNF, resulting in a complete blockade of luciferase signals (fig. 7a), while U73343, an inactive analogue of U73122, at 1 and 10 μM did not have any effects ($p > 0.05$). Wortmannin and U73122 had no effects on the basal luciferase activity (1.09 ± 0.05 - and 1.00 ± 0.24 -fold induction with 5 μM wortmannin and U73122, respectively, over vehicle-treated cells) whereas treatment with thapsigargin resulted in slight increase of the signal in the absence of BDNF (1.44 ± 0.1 - and 1.51 ± 0.11 -fold induction by 0.5 and 2 μM , respectively). On the other hand, treatment with K252a resulted in a U-shape response with a partial inhibition only and subsequent potentiation of BDNF signals at high concentrations of K252a (fig. 7b). Interestingly, at these high concentrations, K252a increased luciferase signals by itself in the absence of BDNF (fig. 7b), suggesting non-specific

CRE-TrkB cells. Luciferase activities were assessed at different time points after BDNF addition. **e** The effect of serum in the luciferase activities. To assess whether the presence of serum alters the BDNF-mediated luciferase responses, cells were incubated with BDNF in culture media containing varying concentrations of serum and the luciferase activities were measured as in the Materials and Methods.

Fig. 7. Pharmacological characterization of the luciferase assay in HEK-CRE-TrkB cells. **a** HEK-CRE-TrkB cells were treated with wortmannin, U73122 or thapsigargin for 15 min at indicated concentrations followed by addition of 100 ng/ml of BDNF and luciferase activities were measured. Cells treated in the absence and presence of BDNF were also included. **b** Dose-dependent effects of K252a. HEK-CRE-TrkB cells were incubated with increasing concentrations of K252a and 15 min later treated or not with 50 ng/ml of BDNF before being processed for the luciferase activity. **c** Effects of ERK/MEK kinase inhibitors PD98059 or U0126

on BDNF-mediated luciferase activity in HEK-CRE-TrkB cells. Cells were pretreated in the absence and presence of inhibitors and then treated in the absence and presence of 100 ng/ml of BDNF before being assayed for luciferase activities. ^a $p < 0.01$ vs. basal; ^b $p < 0.05$ vs. basal; ^c $p < 0.01$ vs. BDNF alone (t test). **d** Dose-dependent activation of the luciferase reporter activities by forskolin. HEK-CRE-TrkB cells were treated with varying concentrations of forskolin and the luciferase activities were determined next day. $EC_{50} = 5.7 \mu$ M.

inhibition of other kinases independent of TrkB. Treatment with PD98059 and U0126, inhibitors of MEK, which had marginal activities on their own, further increased the luciferase activities in all doses tested when combined with BDNF (fig. 7c). In a subsequent experiment, we also

examined the response of compounds that directly affect signal transduction involved in CREB phosphorylation. For example, forskolin, a protein kinase A activator, induced dose-dependent increases of CREB activities in both parental and HEK-CRE-TrkB cell lines (fig. 7d and

unpublished data). The dose responses were similar in both cell lines generating EC₅₀ values of 6.4 and 6.0 μ M in HEK-CRE and HEK-CRE-TrkB cells, respectively. Similarly, we also observed robust dose-dependent luciferase induction by phorbol 12-myristate-acetate (PMA) in both cell lines (9 and 11.8 maximum fold induction over the basal in HEK-CRE and HEK-CRE-TrkB cells, respectively).

Discussion

Potential therapeutic utilities of TrkB agonists are widely reported in various experimental models of human diseases, yet there is no effective drug available in the clinic that directly modulates these systems. From a drug discovery perspective, this provides tremendous opportunities in which one could explore the molecular determinants of the receptor in affecting intracellular activities and lead to a development of a first-in-class therapy where no treatments exist. While full-length NT dimers appear to activate Trk receptors by inducing dimerization, the structural evidence depicting the limited number of spatially restricted contact sites between NT and Trk [6, 33] supports the argument that a small molecule could span the distance necessary to homodimerize the Trk receptors. Precedent for this mode of action may be provided by SB-247464, a small non-peptide compound that displays high specificity and potency for the G-CSF receptor [25]. The biological activity of SB-247464 is indistinguishable from that of the natural ligand, G-CSF, which is 120 amino acid cytokine. That the structure of SB-247464 contains 2-fold rotational symmetry suggests that its agonist activity might be mediated by dimerization of the G-CSF receptor [25].

The aim in this report was to develop a sensitive and simple functional assay to measure Trk activation, which can be implemented in compound screening. We chose TrkB as a prototype of all the NT receptors to validate the assay. As illustrated in figure 1, activation of TrkB is propagated through multiple intracellular signal transduction cascades, which may converge to phosphorylate CREB and induce CRE-dependent transcriptional regulation. CRE-dependent activities have been shown to be important for NT-dependent neuronal survival and synaptic plasticity [19, 34]. Since we had an available CRE-luciferase reporter gene stably expressed in HEK293 cells, we evaluated this system. Transient transfection of TrkB in HEK-CRE cells resulted in a time- and dose-dependent accumulation of a luciferase signal in response to BDNF.

The temporal analysis revealed an initial lag period followed by a gradual increase in the signal, consistent with the luciferase accumulation resulting from induction of transcription. The sensitivity to BDNF and robustness of the signal suggest that this system may be used to analyze the mechanism of BDNF action, including the identification of small molecular weight compounds. However, the distinctive temporal profiles between the receptor activation (in minutes) and reporter activities (in hours) have to be taken into consideration in evaluating the activity of screening compounds, since the compounds' properties such as stability and toxicity could affect the apparent outcome in the reporter activities. Importantly, the protracted incubation time required to attain luciferase signals may select out compounds that are rapidly deactivated or promote toxicity. As our interest is to identify compounds that regulate long-range effects, such as neuroprotection and regeneration, this assay should filter out undesirable compounds.

To permit a thorough analysis, including the possibility of high-throughput screening of small molecules, we produced Trk receptor stable cell lines. One line (HEK-CRE-TrkB clone 22) was chosen for further characterization. We confirmed the expression of TrkB by the migration in Western blots of a band consistent with the apparent molecular weight of full-length TrkB. Radioligand binding studies indicated that TrkB had a sub-nanomolar affinity for [¹²⁵I]BDNF consistent with reported values [30] and a higher density of binding sites than expressed in native tissues [35]. In the luciferase assay, BDNF was highly potent, induced a robust signal, and yielded a Z factor suitable for HTS assay [31]. To directly measure TrkB activation, BDNF-induced autophosphorylation of TrkB was examined. By Western blot analysis, autophosphorylation was significantly inhibited with a selective tyrosine kinase inhibitor K252a. The ranges of effective BDNF doses in both the luciferase assay and for autophosphorylation resulted in comparable EC₅₀ values, which concur with published physiological BDNF concentrations tested in neuronal cultures [29, 36]. In the luciferase assay, the rank order potency for TrkB agonists was BDNF = NT-4/5 > NT-3 and NGF and bFGF were inactive. Alternatively, NGF was highly potent in inducing luciferase activity in a HEK-CRE-TrkA cell line. These results showed that the HEK-CRE cells provided a suitable cell line for high-level expression of Trk receptors.

In another set of experiments, we co-treated HEK-CRE-Trk cells with soluble TrkB-Fc proteins and cognate NT and monitored the luciferase response. We observed that TrkB-Fc led to a dose-dependent inhibition of BDNF-

induced luciferase response in HEK-CRE-TrkB but not of NGF-induced luciferase signal in HEK-CRE-TrkA cells. These results conferred two notions: first, they provided sound evidence on the selectivity of the assay toward TrkB, and second, demonstrated that the assay could also be used to identify selective Trk receptor antagonists, in addition to agonists.

CREB-mediated luciferase transcription is regulated via activation of several intracellular enzymatic pathways downstream of TrkB. As anticipated, treatments of BDNF-stimulated HEK-CRE-TrkB cells with pharmacological inhibitors of these pathways such as wortmannin (inhibits PI3K), thapsigargin (inhibits intracellular calcium pumps), and U73122 (inhibits PLC- γ) resulted in marked reduction of the luciferase readouts. It is important to note that these inhibitors did not simply quench a luciferase signal, as the addition of these inhibitors after the luciferase signal had accumulated did not affect the responses (data not shown). Treatments with K252a, a receptor tyrosine kinase inhibitor, resulted in a U-shape curve, with maximal inhibition occurring at 20 nM, and potentiation of the BDNF response at higher concentrations (>100 nM). We speculated that K252a at high concentrations lost its selectivity (IC₅₀s of 10–30 nM [37]) and consequently raised luciferase signals presumably via altering the phosphorylation status of other proteins independent of TrkB [38, 39], which may warrant further confirmation. A biphasic dose-response has been reported for the ability of K252a to potentiate the NT-3 action on cholinergic neurons and a similar inverted U-shaped curve has been observed with a structural analogue K252b [40]. Finally, treatment with two MEK1 inhibitors (PD98059 and U0126) potentiated the BDNF-induced luciferase signals. The concentrations of these inhibitors used in the study were chosen based on the literature information [29, 41, 42] and were expected to completely inhibit MEK1, although we have not confirmed this independently. It is therefore presumed that in our cellular context these inhibitors modulated other substrates in addition to MEK1, such as other kinases/phosphatases, which directly or in-

directly elevated the BDNF response. This notion is consistent with our observation that PD98059 by itself could increase luciferase signals in the absence of BDNF. A thorough time dependent study may detect MAPK-mediated modulation of CRE activities. Alternatively, it cannot be excluded that BDNF-mediated CREB signaling in our assay is MAPK pathway-insensitive.

While our data validate the assay system as an effective screen for TrkB activity, it needs to be kept in mind that the CRE-mediated reporter activity can also be influenced by compounds that directly affect phosphorylation status of several intracellular kinase cascades, independent of TrkB. It is well exemplified in the study with forskolin and PMA, which induced significant increases in the luciferase activity in both control and TrkB cells. Therefore in order to identify Trk-selective agonists, it is critical to incorporate multiple control experiments to sieve out off-target compounds. To this end, we have established TrkA-expressing cells as well as a parental control clone, which contain the same machinery leading to CRE signaling as the TrkB cell line, while not responding to BDNF. Cross-comparison in these cell systems, therefore, should allow differentiation of potential TrkB agonists from false-positive compounds.

In conclusion, we have developed a highly sensitive and homogenous reporter assay for Trk receptors, which could be implemented in high-throughput format for the screening of compound libraries. Combined with well-designed counterscreening as well as binding and structural analyses, this assay system may provide a powerful approach to identify hits and facilitate early lead optimization of small molecular weight compounds that modulate Trk receptors.

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