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Novel Agonist Monoclonal Antibodies Activate TrkB Receptors and Demonstrate Potent Neurotrophic Activities

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Tyrosine kinase receptor B (TrkB) mediates neurotrophic effects of brain-derived neurotrophic factor (BDNF) to increase neuronal survival, differentiation, synaptic plasticity, and neurogenesis. The therapeutic potential of TrkB activation using BDNF has been demonstrated well in several preclinical models of CNS diseases, validating TrkB as a promising drug target. Therefore, we aimed to develop TrkB-specific receptor agonists by using a monoclonal antibody approach. After generation of hybridoma clones and assessment of their binding and functional activity, we identified five mouse monoclonal antibodies that show highly selective binding to TrkB and that induce robust activation of TrkB signaling. Epitope mapping studies using competition analysis showed that each of the monoclonal antibodies recognizes a unique binding site on TrkB, some of which are distinct from BDNF docking sites. These antibodies behave as true agonists based on their ability to both activate proximal and secondary signaling molecules downstream of TrkB receptors and promote neuronal survival and neurite outgrowth. The binding affinities and the functional efficacy of these antibodies are comparable to those of BDNF, whereas they do not bind to the p75 low-affinity neurotrophin receptor at all. Therefore, they could represent novel reagents to explore the pathophysiological roles of TrkB and its potential therapeutic utility in treating CNS disorders.

Key words: **neuroprotection; neurite outgrowth; binding; agonists; monoclonal antibodies; phosphorylation**

Introduction

Tyrosine receptor kinases are a family of single transmembrane glycoproteins that mediate various trophic effects of the neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. A large body of evidence demonstrates crucial roles of neurotrophins in a wide spectrum of neuronal responses such as neurogenesis, differentiation, and neurite growth (Connor and Dragunow, 1998). In particular, BDNF, a cognate ligand to tyrosine kinase receptor B (TrkB), has been shown to exert strong survival and neuroprotective effects on the neurons of the CNS. For example, BDNF protects hippocampal neurons from glutamate toxicity (Lindholm et al., 1993) and rescues cerebellar neurons from programmed cell death (Leeds et al., 2005). In animal studies, BDNF has been shown to reduce ischemic injury (Schabitz et al., 2000; Kurozumi et al., 2004) and to improve functional recovery and postinjury regeneration (Koda et al., 2004).

These preclinical data strongly support the rationale for clinical evaluation of BDNF as a therapeutic agent for various neurological disorders. However, the outcome of several clinical trials using recombinant BDNF has been disappointingly negative (Ochs et al., 2000; Beck et al., 2005), and it is potentially because of the short *in vivo* half-life of BDNF. In addition, BDNF also binds to the p75 neurotrophin receptor (p75NTR), a promiscuous NTR that is known to activate cell death pathways via recruitment of multiple adaptor proteins (Chao, 1994; Friedman and Greene, 1999). In fact, BDNF has been shown to exacerbate cytotoxicity when TrkB expression is not present (Friedman and Greene, 1999), which might have also contributed to the lack of efficacy in the clinic. To date, no examples of exogenous agents exist that act as potent and selective *in vivo* agonists of TrkB.

TrkB is one of the most widely distributed NTRs in the brain, whose expression is high in such areas as the neocortex, hippocampus, striatum, and brainstem (Muragaki et al., 1995; Shelton et al., 1995). It is a multidomain transmembrane protein that consists of an extracellular ligand binding domain, a transmembrane region, and an intracellular tyrosine kinase domain (McDonald and Chao, 1995). BDNF binding to TrkB induces autophosphorylation of TrkB and, subsequently, phosphorylation of several mediator kinases, including extracellular signal-regulated kinase [mitogen-activated protein kinase (MAPK)], phosphatidylinositol 3-kinase/Akt, phospholipase C-γ, and their downstream targets (Middlemas et al., 1994; Encinas et al., 1999). The binding of neurotrophins to Trk receptors has been shown to induce receptor dimerization that leads to conformational changes and activation of intracellular signal transduction pathways (Jing et al., 1992). In the case of TrkA, a receptor for NGF, it was shown that receptor cross-linking of TrkA by use of polyclonal or monoclonal antibodies (mAbs) mimicked the biological effects of NGF (Clary et al., 1994; LeSauteur et al., 1996). These Abs appear to share binding sites with NGF as evidenced by competition with NGF binding and the inhibition of NGF-mediated neurite outgrowth. The role of other extracellular

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regions of TrkA in receptor dimerization and signaling activation remains elusive.

In this study, we present novel TrkB-selective mAbs with diverse binding epitopes that fully recapitulate the trophic activities of BDNF. They effectively activate TrkB signaling and promote neuronal survival and neurite outgrowth, thus representing potent agonists of TrkB.

Materials and Methods

Immunization schedules. Five 8-week-old female BALB/c mice were immunized with10 μg of recombinant human TrkB-extracellular domain (rhTrkB- ECD) (R & D Systems, Minneapolis, MN) and 1 μg of recombinant mouse TrkB-ECD (rmTrkB) (R & D Systems) that were premixed with complete Freund's adjuvant. The animals were immunized four times, biweekly. Mice were bled 1 week after the last immunization, and the Ab responses in the antisera were evaluated. Three mice were chosen for hybridoma preparation. They received one additional boost of 10 μg of rhTrkB and 1 μg of rmTrkB 3 d before the cell fusion.

Generation of mouse anti-TrkB mAbs. Splenocytes from three BALB/c mice immunized with rhTrkB and rmTrkB were fused with mouse myeloma cells (P3X63Ag8.653; American Type Culture Collection, Manassas, VA) at a 4:1 ratio using 50% polyethylene glycol (MW 1500; Roche Diagnostic Systems, Somerville, NJ). After fusion, cells were seeded and cultured in 96-well plates at 1×10^5 cells/well in the RPMI 1640 selection medium containing 20% fetal bovine serum (FBS), 5% Origen (IGEN International, Gaithersburg, MD), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 1× hypoxanthine-aminopterin-thymidine (Sigma, St. Louis, MO). Hybridoma supernatants were screened for binding to human TrkB (hTrkB) by ELISA using rhTrkB-Fc (R & D systems) and by fluorescence-activated cell sorting (FACS) analysis on human embryonic kidney 293 (HEK293) cells stably expressing hTrkB (Zhang et al., 2006). Hybridoma supernatants with a positive binding signal were also tested for functional agonist activity using a luciferase reporter assay (see below, Luciferase reporter assay). Selected hybridomas were subcloned four times by serial dilution and once by FACS. Conditioned media were harvested from the stable hybridoma cultures, and Igs were purified using Prosep-A Montage Ab purification spin columns (Millipore, Bedford, MA). The Ig class of mAb was determined with a mouse mAb isotyping kit (IsoStrip; Boehringer Mannheim, Indianapolis, IN).

Cell culture. HEK293 cells stably expressing full-length hTrkB and 3xCRE_luc (3x cAMP-responsive element-Luc vector; Mercury Signal System; Clontech, Palo Alto, CA), referred to as HEK-TrkB, were described previously (Zhang et al., 2006). These cells were grown in standard DMEM containing 10% fetal calf serum supplemented with 1% penicillin/streptomycin, 250 μg/ml geneticin/G418, 2 μg/ml puromycin, and 350 μg/ml hygromycin. HEK-TrkA and HEK-TrkC cells were cultured in the same standard media as for HEK-TrkB. Chinese hamster ovary (CHO)-p75NTR stable cells were grown in α-medium (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal calf serum and 20 nM utx-methotrexate. Human neuroblastoma SH-SY5Y cells were grown in DMEM:F-12 (1:1) supplemented with 2 mM L-glutamine, 15% fetal calf serum, and 1% penicillin/streptomycin. Primary neuronal cultures were prepared from rat and mouse cerebella and rat cortex. After dissection of the cerebellum from postnatal day 5–7 animals, tissues were cut into small pieces and enzymatically digested for 30 min using a papain dissociation kit (Worthington, Freehold, NJ). After dispersion by gentle trituration and centrifugation at 300 × *g* for 5 min, dissociated cerebellar granule neurons (CGNs) were reconstituted in a standard Neurobasal growth medium containing B27 supplement, 0.5% FBS, 0.5 mM L-glutamine, and 25 mM potassium chloride and plated in multiwell Biocoat plates precoated with poly-D-lysine (BD Biosciences, Franklin Lakes, NJ). Rat cortical neurons were isolated from postnatal day 1–2 rats using the same protocol described above for CGNs and plated in a B27-supplemented Neurobasal medium. All cultures were maintained at 37°C in 5% CO_2 and 95% air.

ELISA. Binding of hybridoma supernatants and purified Abs to hTrkB was evaluated using ELISA. Ninety-six-well plates (Maxisorp; Nunc, Naperville, IL) were coated with either 1 μg/ml rhTrkB (R & D systems) or rmTrkB (R & D systems) and incubated overnight at 4°C. After washing and blocking with PBS containing 1% BSA and 0.05% Tween 20, 100 μl of sample (diluted immune serum, hybridoma supernatants, or purified Abs, as indicated in the figures) was added and incubated for 1 h at room temperature. The plates were washed, and the bound anti-TrkB Abs were detected using peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL), followed by incubation with the substrate TMB (BioFX Laboratories, Owings Mills, MD) for color development. Absorbance values were determined at 450 nm in a spectrophotometer. The concentrations of mAbs were determined using peroxidase-labeled goat anti-mouse IgG (Fcγ) (Pierce) and a standard curve generated by a purified isotype-matching mouse IgG.

FACS analysis. Cells were suspended in PBS containing 1% BSA at a density of 4×10^5 cells/ml. Cells were incubated with 100 μl of suspended sample (diluted immune sera, hybridoma supernatants, or purified Abs) for 30 min at 4°C. After washing, cells were incubated with phycoerythrin (PE)-labeled goat anti-mouse IgG, $F(ab')_2$ (Dako, Carpinteria, CA) for 30 min at 4°C in the dark. Cell-associated fluorescence signals were measured by a FACScan flow cytofluorometer (Becton Dickinson, Heidelberg, Germany) using 5000 cells per treatment. Propidium iodide was used for identification of dead cells, which were excluded from the analysis.

Determination of relative Ab-binding epitopes on hTrkB. To determine whether TrkB mAbs affect BDNF binding to TrkB, we conducted competition ELISA binding assays. Ninety-six-well plates were coated with 0.3 μg/ml BDNF (R & D Systems) overnight at 4°C. After washing and blocking the wells with PBS containing 1% BSA and 0.05% Tween 20, 100 μl of preincubated mixtures containing Ab samples (diluted immune sera, hybridoma supernatants, or purified Abs) and rhTrkB-Fc (150 ng/ml) was added to each well and incubated for 1 h at room temperature. Plates were washed, and BDNF-bound rhTrkB-Fc was detected using Fcγ (Pierce), followed by incubation with the substrate TMB (BioFX Laboratories) for color development. Binding of rhTrkB-Fc to BDNF without any competing Abs or with diluted preimmune serum was used as a control and defined as 100% binding.

We used similar competition approaches to determine the relative binding epitopes between each pair of selected mAbs. First, 1 μg/ml purified mAb was added to each well of a 96-well plate and defined as a "competing" Ab. Subsequently, 100 μl of one of the mAbs (20 μg/ml) premixed with rhTrkB-Fc (150 ng/ml), referred to as "prebound" Ab, was added to the wells and incubated for 1 h at room temperature. After washing, the amount of rhTrkB-Fc remaining on the plate that was bound to the competing Abs was determined using Fcγ, as described above.

Luciferase reporter assay. Induction of luciferase was measured to assess the agonistic activities of TrkB mAbs, as described previously (Zhang et al., 2006). In brief, HEK-TrkB cells were plated at a density of 1.5×10^5 cells/ml in 96-well plates in standard DMEM. The next day, cells were stimulated with BDNF or Abs, as indicated in the figures, and the luciferase activity was measured after 16 h of incubation using the Steady-Glo assay kit (Promega, Madison, WI). After replacement of culture media with 100 μl of PBS, an equal volume of Steady-Glo assay reagent was added to induce cell lysis and the plates were shaken on a Titer Plate Shaker (Lab Line Instruments, Melrose Park, IL) for 5 min. The luminescence signal was measured using TopCount NXT (Packard, Meriden, CT), and the results were presented as fold increases over basal activity.

TrkB autophosphorylation assay/Western blot. HEK-TrkB cells were plated in 24-well plates at 2×10^5 cells/well in standard DMEM. The next day, cells were incubated in serum-free DMEM for 90 min before stimulation with BDNF or Abs at indicated concentrations for 30 min at 37°C. Stimulation was terminated by removal of the medium followed by one wash with PBS. Cells were lysed in 100 μl of Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and heated to 95°C. Cellular lysates were filtered through a QiaShredder column (Qiagen, Valencia, CA), and 20 μl of samples was resolved on 4–12% Bis-Tris gels (Invitrogen) and processed for Western blot analysis. For testing of primary neurons, rat CGNs were plated in six-well plates at a density of 1×10^6 cells/well. Cells were serum deprived for 2 h, treated with BDNF or Abs for 30 min, and lysed in 100

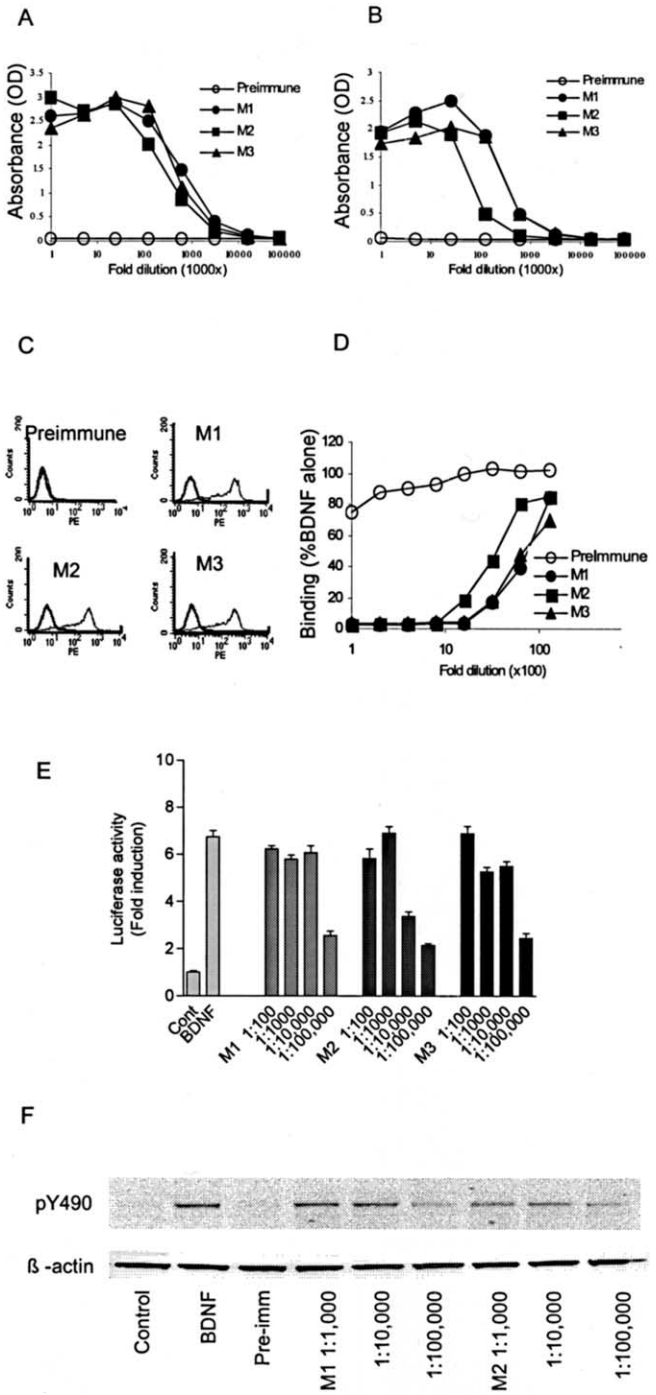

Figure 1. Immunization with hTrkB and mTrkB elicited cross-reactive Ab responses with agonistic activities. Immune sera from three BALB/c mice (M1, M2, and M3) were obtained 7 d after the fourth immunization. ***A***, ***B***, Immune sera were diluted in PBS containing 1% BSA and subjected to ELISA to examine their binding activities to hTrkB (***A***) or mTrkB (***B***), as described in Materials and Methods. Preimmune sera were used as a negative control. ***C***, Immune or preimmune sera at a 1:1000 dilution were incubated with HEK-TrkB cells (gray traces) or parental HEK cells (black traces), followed by secondary incubations with PE-labeled goat anti-mouse Abs, and the staining intensities were analyzed using FACS. ***D***, Various dilutions of immune or preimmune sera were incubated with rhTrkB-Fc (150 ng/ml) for 30 min and added to ELISA plates precoated with BDNF. After wash, the amount of rhTrkB-Fc bound to BDNF was measured using peroxidase-conjugated goat anti-human IgG. The binding response of rhTrkB-Fc and BDNF without immune sera was defined as a total binding and was expressed as 100%. The addition of preimmune serum had no significant effect on rhTrkB-Fc binding to BDNF, whereas immune sera competed with this interaction in a dose-dependent manner. ***E***, HEK-TrkB cells in 96-well plates were stimulated with BDNF (50 ng/ml) or immune sera at various dilutions, and the luciferase activities were measured 16 h later. All three immune sera (M1, M2, and M3) induced dose-dependent increases of the luciferase activities. Data are presented as mean fold induction over the basal level ± SEM from triplicate samples. Cont, Control. ***F***, HEK-TrkB cells were treated with BDNF (50 ng/ml), a preimmune serum (pre-imm; 1:1000), or immune sera at indicated dilutions for 30 min. Equal amounts of cell lysates were resolved in the SDS-PAGE gel, and phosphorylation levels of TrkB were detected using anti-pY490 as described in Materials and Methods. β-Actin was used to verify equal loading of proteins in each lane. The blot shown here represents one of the two independent experiments with similar results.

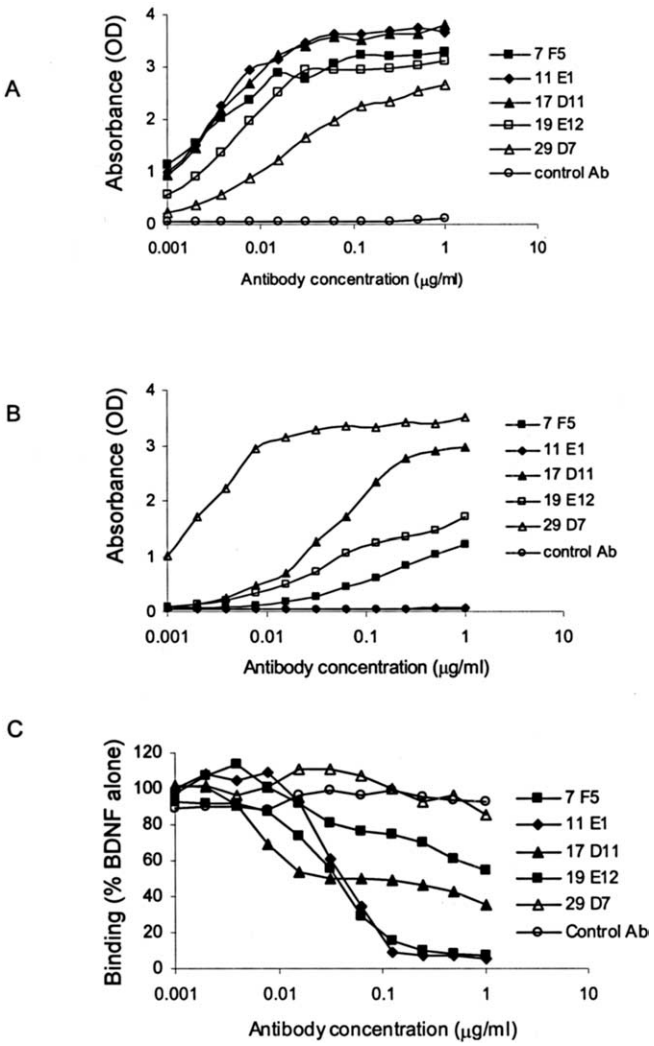

Figure 2. Binding properties of TrkB mAbs. ***A***, ***B***, Dose–response curves demonstrating the binding activities of five mAbs to hTrkB (***A***) and mTrkB (***B***), as determined by ELISA. ***C***, Various concentrations of TrkB mAbs were incubated with rhTrkB-Fc (150 ng/ml) for 30 min, and the mixture was added to ELISA plates precoated with BDNF. After wash, the amount of rhTrkB-Fc bound to BDNF was measured using peroxidase-conjugated goat anti-human IgG. The binding of rhTrkB-Fc to BDNF in the absence of any mAbs was determined as a total binding and was expressed as 100%. In all assays, isotype-matched mouse Abs (mIgG1, mIgG2a) were used as a negative control.

μl of RIPA buffer. Protein concentrations were determined, and 20 μg of total proteins was loaded on the 4–12% Bis-Tris gel (Invitrogen). After transfer to the nitrocellulose membranes, immunoblotting was performed using PY490 phospho-Trk-specific Ab (9141, 1:100; Cell Signaling, Beverly, MA), anti-phospho-MAPK Ab (1:2000; Cell Signaling), anti-MAPK (1:5000; Cell Signaling), anti-phospho-Akt (1:1000; Cell Signaling), or anti-β-actin (1:3000; Sigma), followed by incubation with HRP-conjugated secondary Abs (Invitrogen). The signal was developed using the ECL plus kit (Amersham Biosciences, Piscataway, NJ).

Neurite outgrowth assay. For the neurite outgrowth assay, SH-SY5Y

neuroblastoma cells were plated in 96-well tissue culture plates at a density of 4×10^3 cells/well and incubated with 10 μM all-trans-retinoic acid (RA) to induce neuronal differentiation. On the third day, the medium was replaced with fresh growth medium with or without BDNF and/or testing Abs, as indicated in the results. After an additional 3 d in culture, cells were fixed by IC-Fix (Biosource, Camarillo, CA) for 30 min at room temperature and also processed for immunostaining of β-tubulin III. First, cells were permeablized by brief incubation in 0.2% Triton in PBS (TPBS). Subsequently, samples were incubated in 1.5% normal goat serum (NGS) in TPBS for 30 min to block nonspecific binding, followed by incubation with anti-β-tubulin III mAb (TuJ1, 1:1000; Covance, Berkeley, CA) in 1.5% NGS/TPBS. TuJ1 signal was detected using Alexa 488 mouse anti-goat Abs (1:500; Invitrogen), and neurite outgrowth was analyzed using Cellomics (Pittsburgh, PA) ArrayScan. For quantification, ~800 cells were analyzed from each well, and typically each condition was run in quadruplicate.

For the evaluation of neurite-promoting effects of Abs in primary neurons, rat and mouse CGN cultures were seeded at a density of 1.2×10^4 cells/well in 96-well plates precoated with poly-D-lysine (BD Biosciences). Cells were treated with BDNF or testing Abs for 24 h, fixed with IC-Fix for 30 min at room temperature, and processed for TuJ1 immunostaining as described above.

Cell survival assay. SH-SY5Y cells were plated in 96-well plates at 1×10^4 cells/well and incubated with RA (10 μM) to induce neuronal differentiation. After 3 d, the culture medium was switched to a standard growth medium without serum (serum-free DMEM) supplemented with BDNF, testing Abs, or a vehicle. After an additional 2 d in culture, cell viability was measured by MTT conversion using the CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega) according to the manufacturer's protocol.

Neuroprotection assay. Rat or mouse CGNs were plated in 96-well Biocoat plates precoated with poly-D-lysine (BD Biosciences) at a density of 7.3×10^4 cells/well. Twenty-four hours after plating, cells were subjected to potassium serum deprivation (KSD) injury, which is known to result in significant death of CGNs (Skaper et al., 1998). Some sister cultures were treated with BDNF or testing Abs under KSD conditions to monitor their neuroprotective efficacies. After a 24–96 h incubation, cell viability was measured using the CellTiter 96 Cell Proliferation Assay kit (Promega). Rat cortical neurons were grown in poly-D-lysine-coated 96-well plates (BD Biosciences) in a Neurobasal medium supplemented with B27 at a density of 6.5×10^4 cells/well. Cells were treated with indicated concentrations of a TrkB Ab or a vehicle for 48 h, and cell viability was measured using the CellTiter 96 Cell Proliferation Assay kit (Promega).

Table 1. Characterization of anti-TrkB mAbs

	ELISA binding		BDNF competition
mAb	hTrkB [ED_{50} (nM)]	mTrkB [ED_{50} (nM)]	IC_{50} (nM)
7F5	0.016	Weak binding	0.3
11E1	0.02	ND	0.3
17D11	0.02	0.3	Partial blocking
19E12	0.031	Weak binding	Partial blocking
29D7	0.12	0.013	ND

For determination of binding avidities of mAbs to TrkB, a series of concentrations of purified Abs was incubated with rhTrkB or rmTrkB coated onto 96-well plates. After a 1 h incubation period, plates were washed and the amounts of Abs bound to TrkB were measured using peroxidase-conjugated secondary Abs. For binding competition with BDNF, preincubated mixtures of mAbs with rhTrkB-Fc were added to ELISA plates coated with BDNF. After a 1 h incubation period, BDNF-bound rhTrkB-Fc was detected using peroxidase-conjugated goat anti-human IgG. ND, Not detectable.

Table 2. Mapping of relative Ab-binding epitopes

	Competing mAb					
Prebound mAb	7F5	11E1	17D11	19E12	29D7	Control mAb[a]
7F5	+	+/−	−	−	−	−
11E1	+	+	−	−	+/−	−
17D11	−	−	+	+/−	−	−
19E12	−	−	+	+	−	−
29D7	−	−	−	−	+	−
Control mAb[a]	−	−	−	−	−	N/A

A preformed complex of each mAb (20 μg/ml) with rhTrkB-Fc (150 ng/ml) was added to each well of 96-well plates coated with competing Abs. After a 1 h incubation period, plates were washed with PBS and the amounts of rhTrkB-Fc remaining in the wells were quantified using peroxidase-conjugated goat anti-human IgG. +, Complete competition; +/−, partial competition; −, no competition; N/A, not applicable.

[a]Isotype-matched mouse mAb.

Results

Evaluation of Ab responses from TrkB-immunized mice

We wanted to raise Abs that cross-react with hTrkB and mouse TrkB (mTrkB), which would then allow us to assess their activities in both experimental models and human systems. Therefore, we used a mixture of hTrkB and mTrkB proteins as immunogens. After a series of immunizations, immune bleeds were drawn for initial evaluation of Ab response to TrkB. All immunized mice generated high titers against rhTrkB and rmTrkB, as determined by ELISA (Fig. 1*A*,*B*). These immune bleeds also bound to membrane-anchored full-length hTrkB expressed on the surface of HEK-TrkB cells, as determined by FACS analysis (Fig. 1*C*). We then questioned whether the binding sites of these Abs overlapped with the BDNF docking site on TrkB. We immobilized BDNF on the ELISA plate and performed competitive binding using rhTrkB-Fc pre-equilibrated with immune sera. Immune sera at high concentrations completely blocked the binding of BDNF to rhTrkB-Fc (Fig. 1*D*). However, with increasing dilution of immune sera, we detected displacement of immune sera with BDNF, allowing the binding of BDNF to rhTrkB (Fig. 1*D*). Although we observed dose-dependent binding responses of immune sera from all three mice, immune serum from mouse 2 in particular showed a binding curve clearly left-shifted from those of mice 1 and 3 (Fig. 1*D*), suggesting it might contain fewer Abs against the BDNF docking site on TrkB or the epitopes important for TrkB–BDNF interaction. Interestingly, immune sera from mice 1 and 3, which displayed higher binding potencies to TrkB as shown in Figure 1, *A* and *B*, also appeared to interfere more with BDNF binding to rhTrkB than mouse 2 did. None of the preimmune sera showed evidence of TrkB binding in either ELISA or FACS assay (Fig. 1*A*–*D*).

We then tested whether our TrkB binding immune sera were functionally agonistic. The luciferase reporter assay using HEK-TrkB has been shown to reflect activation of TrkB in a receptor- and ligand-selective manner (Zhang et al., 2006). Thus, TrkB-selective ligands, BDNF and NT-4, induced robust increases in the luciferase signal, whereas other growth factors including NGF and basic FGF had no effects (data not shown). Using this assay, we observed that treatment of cells with immune sera significantly increased the luciferase signal in a dose-dependent manner (Fig. 1*E*). Again, immune sera from mice 1 and 3 enhanced luciferase activity with higher potency than mouse 2, consistent with their higher binding to TrkB as shown in Figure 1*A*. To confirm that the immune sera indeed activated TrkB, we examined the autophosphorylation of TrkB receptors using Western blot analysis. Treatment of HEK-TrkB cells with BDNF induced a marked increase in the protein band intensity detected by an Ab specifically recognizing phosphorylated Trk (PY490) (Fig. 1*F* and our unpublished data). Also, incubation with immune sera resulted in dose-dependent increases of TrkB phosphorylation (Fig. 1*F*). Cell lysates harvested after treatment with preimmune

bleeds were devoid of any phosphorylation signals similar to the control.

Production and characterization of mAbs

Splenocytes from three mice were fused with mouse myeloma cells, and the supernatants from the resultant hybridomas were screened for TrkB binding using ELISA and FACS. We identified clones showing robust binding to rhTrkB, some of which cross-reacted with rmTrkB (data not shown). The agonist activities of all of the binding clones were tested in a luciferase assay, and the agonist clones were subjected to additional subcloning by serial dilution and FACS to achieve monoclonality. These steps led us to isolate a final set of five unique mAbs, as described below.

Illustrated in Figure 2, *A* and *B*, are the dose-dependent binding activities of mAbs to hTrkB and mTrkB. All five Abs showed high ELISA binding avidities to rhTrkB with ED_{50} values of $10^{-10\sim-11}$ M, as summarized in Table 1. We also confirmed their binding activities to membrane-associated hTrkB expressed on the cell surface using FACS analysis (see Fig. 4*C*). We then evaluated whether our Abs could compete with BDNF binding to TrkB. Results from ELISA analysis revealed varying degrees of competition by each Ab with BDNF binding to rhTrkB (Fig. 2*C*). Clones 7F5 and 11E1 effectively blocked TrkB–BDNF interaction with an IC_{50} of 3×10^{-10} M, whereas clones 17D11 and 19E12 were able to only partially interfere with BDNF binding, even at the highest concentrations tested (1 μg/ml). Notably, clone 29D7 did not affect BDNF docking to TrkB at all. Furthermore, our Abs also varied in their ELISA binding avidities to mTrkB. Clones 17D11 and 29D7 bound rmTrkB with high avidities (ED_{50} = $10^{-10\sim-11}$ M), whereas the rest of our Abs showed very weak or no binding to rmTrkB (Fig. 2*B*). The variable binding characteristics of these Abs suggest that they each might recognize a unique epitope on TrkB.

Next, we evaluated the relative binding epitopes of our Abs by examining the capability of each Ab to block the binding of other Abs to rhTrkB. Our analysis showed that clones 7F5 and 11E1 might share binding epitopes, as do clones 17D11 and 19E12. The binding site of clone 29D7 appeared unique, not overlapping with the other Abs in our panel (Table 2).

The agonist activities of purified Abs were examined using the luciferase assay. Purified Abs resulted in dose-dependent increases in luciferase signal with EC_{50} values of ~0.03 μg/ml (~10^{-10} M) (Fig. 3*A*). The maximum signal induced by Abs was sixfold to eightfold higher than the background level. Sister cultures treated with BDNF as a positive control showed increases in activity by 5- and 6.2-fold over background with 100 and 200 ng/ml BDNF, respectively, demonstrating comparable signal windows with Ab treatments. Induction of luciferase activity by TrkB Abs was completely prevented by coincubation with hrTrkB-Fc protein (Fig. 3*B*), also confirming that Ab-mediated signal activation requires functional TrkB.

To provide direct evidence that the luciferase signaling was indeed mediated through TrkB, we took a proximal measure of TrkB activation by assessing autophosphorylation of TrkB. HEK-TrkB cells treated with agonist mAbs were evaluated in a Western blot analysis using an anti-phospho-Trk Ab, PY490. The immunoblots in Figure 3*C* clearly show that treatment with agonist Ab led to robust TrkB phosphorylation. The Ab responses were effectively blocked by cotreatment with K252a, a receptor tyrosine kinase inhibitor, also confirming the selective involvement of TrkB rather than a general induction of kinase pathways (Fig. 3*C*). Treatment with our Abs also induced robust phosphorylation of MAPK and Akt kinases, downstream signaling mediators

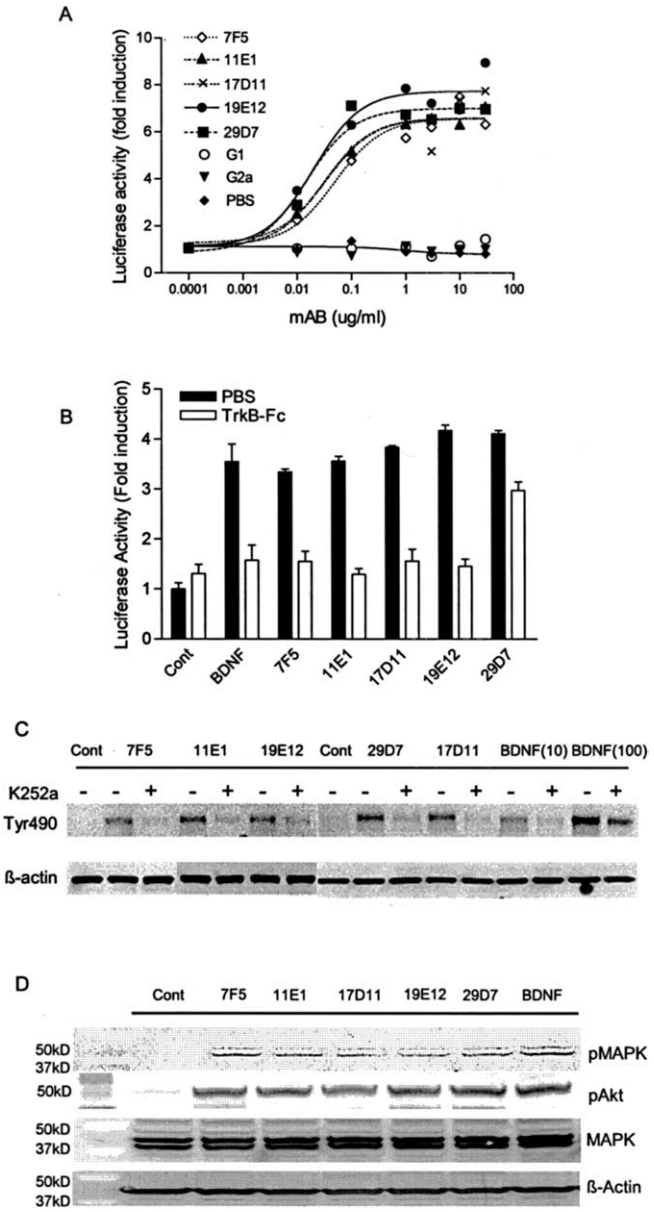

Figure 3. Activation of TrkB-dependent luciferase signaling and phosphorylation cascades by TrkB mAbs. ***A***, HEK-TrkB cells in 96-well plates were treated with purified TrkB mAbs, isotype-matching control Abs (G1, mIgG1; G2a, mIgG2a), or a vehicle (PBS), and the accumulated luciferase activities were measured after 16 h. Data are presented as mean fold induction over the basal level ± SEM from triplicate samples. ***B***, HEK-TrkB cells were treated with TrkB mAb (0.1 μg/ml) or BDNF (25 ng/ml) in the presence or absence of hrTrkB-Fc (2 μg/ml), and the induction of luciferase activities was assessed as in ***A***. ***C***, HEK-TrkB cells were stimulated with each mAb (1 μg/ml) or with BDNF (10 or 100 ng/ml) in the presence (+) or absence (−) of K252a. Cell lysates were resolved in the SDS-PAGE gel, and phosphorylation levels of TrkB were detected using anti-pY490 as described in Materials and Methods. β-Actin was used to verify equal loading of proteins in each lane. All mAbs as well as BDNF led to robust phosphorylation of TrkB, which was effectively blocked by K252a. ***D***, HEK-TrkB cells were stimulated with indicated mAbs (1 μg/ml) or BDNF (100 ng/ml), and the cell lysates were analyzed for phosphorylation of MAPK and Akt kinases using phospho-specific Abs. The same samples were also probed for a total MAPK protein and β-actin to verify equal loading. Cont, Control.

of TrkB cascades, which presumably would have led to cAMP response element-binding protein (CREB)-mediated transcriptional activation and luciferase induction (Fig. 3*D*).

To demonstrate that the activities of these mAbs are selective for TrkB, we used HEK cells stably expressing either TrkA or TrkC and followed luciferase activation. Treatment of HEK-

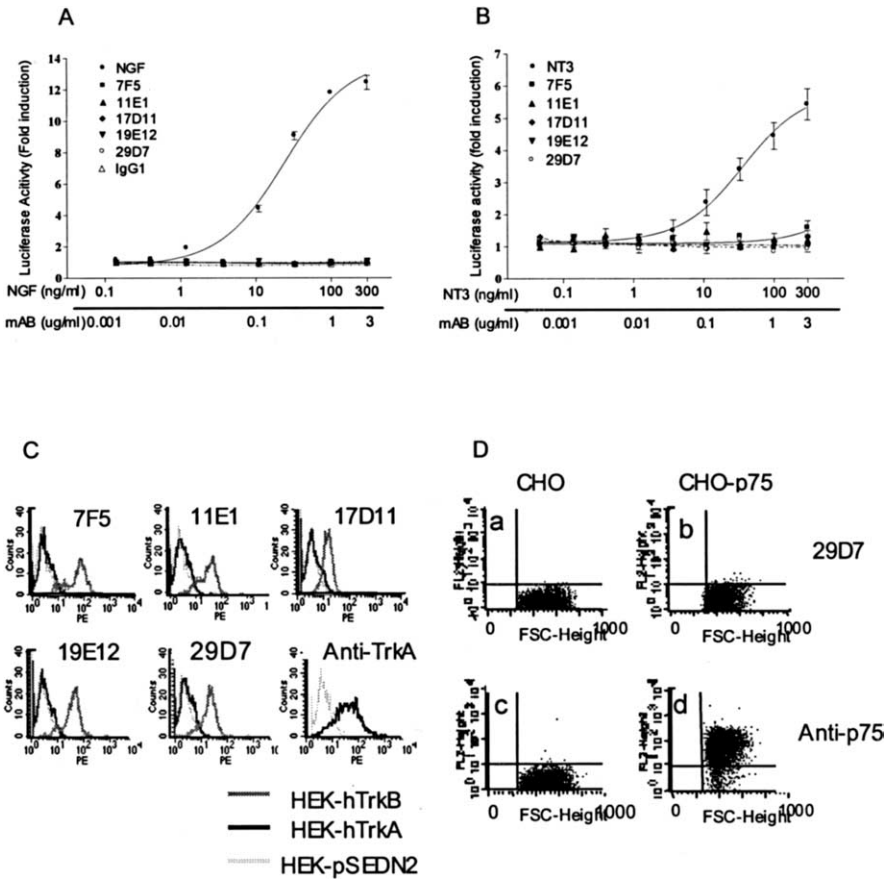

Figure 4. TrkB mAbs are not cross-reactive with TrkA, TrkC, or p75NTR. ***A***, ***B***, HEK-TrkA (***A***) or HEK-TrkC (***B***) cells were seeded in 96-well plates and stimulated with NGF, NT-3, TrkB mAbs, or a control Ab as indicated in the figures. Sixteen hours later, the accumulated luciferase activities were detected as a measure of receptor activation. Data are presented as the mean fold induction over the basal level ± SEM from triplicate samples, and the experiment was repeated at least twice. ***C***, Purified TrkB mAbs diluted at 1:1000 were added to HEK-TrkB (gray traces), HEK-TrkA (black traces), or pSEDN2 vector-transfected (HEK-pSEDN2; light gray traces) cells. Cellular binding of mAbs was detected using PE-labeled goat anti-mouse Abs, and the staining intensities were analyzed using FACS. TrkB mAbs specifically bound to TrkB-expressing cells but not to TrkA- or vector-expressing cells. An anti-hTrkA Ab was used as a positive control to demonstrate its binding to HEK-TrkA cells. ***D***, A 29D7 TrkB mAb or an anti-p75NTR Ab (0.5 μg/ml) was incubated with parental (CHO; ***a***, ***c***) or p75NTR-expressing (CHO-p75; ***b***, ***d***) cells. After a 30 min incubation, binding signals were detected using PE-labeled goat anti-mouse Abs, and cell-associated fluorescence was detected by FACS analysis.

TrkA or HEK-TrkC cells with BDNF had no effect on the luciferase activities (data not shown). In contrast, treatment of HEK-TrkA cells with NGF and HEK-TrkC cells with NT-3 resulted in dose-dependent increases in their luciferase activities, suggesting the intactness of the cellular signaling system (Fig. 4*A*,*B*). Remarkably, none of our TrkB-specific Abs showed any activity in these assays, indicating that they did not cross-react with TrkA or TrkC. FACS analysis using TrkA cells also confirmed lack of binding of TrkB mAbs to TrkA (Fig. 4*C*). In addition, we tested binding activities of TrkB Abs on p75NTR using a recombinantly prepared CHO-p75NTR cell line. As shown in Figure 4*D*, there was no detectable binding of 29D7 to either parental or p75NTR-expressing cells. Similar results were observed with other TrkB Abs (data not shown). In contrast, a known p75NTR-binding Ab (R & D Systems) increased the fluorescence signal when added to CHO-p75NTR cells, as expected (Fig. 4*D*).

Functional activities of mAbs

It is known that BDNF promotes neurite outgrowth and survival of TrkB-expressing neuronal cells. We therefore tested whether TrkB mAbs could mimic the biological effects of BDNF via activation of endogenous TrkB receptors. Because our Abs bound both hTrkB and mTrkB, we first used human neuroblastoma SH-SY5Y cells, which are known to express TrkB receptors after RA-induced neuronal differentiation (Kaplan et al., 1993; Encinas et al., 1999). Consistent with previous reports (Encinas et al., 1999), we observed that the addition of BDNF promoted neurite outgrowth as demonstrated by increases in neurite length and the number of branch points (Fig. 5). All of our TrkB Abs also significantly increased neurite outgrowth of differentiated SH-SY5Y cells in a dose-dependent manner, with maximum responses comparable or even superior to BDNF (Fig. 5*A*,*B*), as shown in representative pictures (Fig. 5*C*). Subsequent full dose–response studies determined the neurite outgrowth EC_{50} of Abs to be between 0.05 and 0.5 nM (Table 3).

In another set of experiments, SH-SY5Y cells were treated with RA to induce neuronal differentiation and were then subsequently challenged with serum deprivation. This resulted in the death of differentiated SH-SY5Y neurons (Encinas et al., 1999; Iglesias et al., 2003), which could be partially rescued by BDNF treatment (Fig. 6). Treatment with TrkB Abs also protected these cells from serum deprivation-induced injury in a dose-dependent manner. Clone 11E1 was even more potent than BDNF in this assay (Fig. 6).

Among the five Abs, we selected two clones, 29D7 and 17D11, which also bind to rmTrkB, as demonstrated above (Fig. 2). Therefore, we wanted to test their functional activities in rodent primary neuronal cultures. We isolated CGN from rats and mice and first confirmed that BDNF increased neurite outgrowth of cultured neurons as reported in the literature (Fig. 7*A*,*B*). We then tested the neurite-promoting effects of TrkB Abs. Clone 29D7 increased the total neurite length and neurite branching of both cerebellar neurons in a dose-dependent manner (Fig. 7*A*,*C*,*D*). Consistent with this efficacy in neuritogenesis, treatment with 29D7 also afforded significant protection of CGN against apoptotic death induced by potassium and serum deprivation. Thus, 29D7 increased neuronal survival by 70% at 24 h after injury (88% viability by 29D7 treatment vs 60% in control) (Fig. 7*E*). Subsequent longer-term studies demonstrated the effectiveness of these treatments in promoting neuronal viability up to 96 h, resulting in 50, 22, and 12% increases in survival at 48, 72, and 96 h, respectively, compared with control (Fig. 7*F*). In contrast, clone 17D11 did not show any functional efficacy in neuronal cultures from either rats or mice, despite our ELISA data that showed robust binding to mrTrkB (ED_{50} = 0.3 nM) (Table 1). To also confirm that 29D7 is capable of activating endogenous TrkB, we measured phosphorylation of TrkB as well as activation of intracellular signaling molecules. After a 30 min treatment of CGN with 29D7, there were clear increases in the

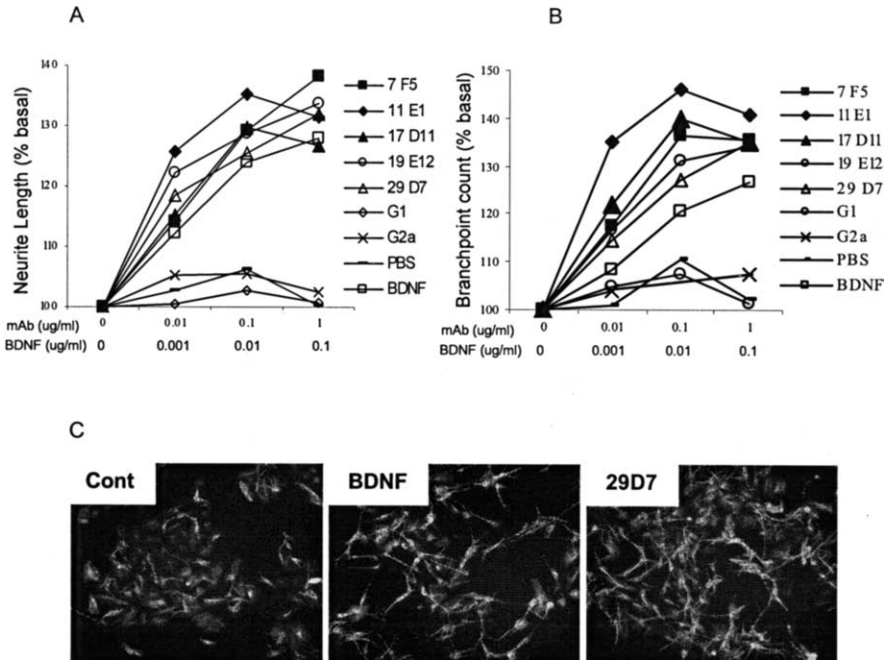

Figure 5. TrkB mAbs promote neurite outgrowth of differentiated SH-SY5Y cells. SH-SY5Y cells were plated in 96-well tissue culture plates and incubated with 10 μM all-trans-RA to induce neuronal differentiation. Three days later, the cells were replenished with fresh growth medium containing BDNF or testing Abs. After an additional 3 d in culture, cells were fixed and stained with anti-β-tubulin III (TuJ1) to trace neurite outgrowth using Cellomics ArrayScan. ***A***, Neurite length, indicating the average of total neurite length for all TuJ1-positive cells, was analyzed. ***B***, Branchpoint counts indicating the average number of branch points for all TuJ1-positive cells analyzed. ***C***, Representative images of TuJ1 staining of SH-SY5Y cells after 3 d treatments with a vehicle control, BDNF (10 ng/ml), or a TrkB Ab 29D7 (0.1 μg/ml). Cont, Control.

Table 3. TrkB Ab-induced neurite outgrowth in differentiated SH-SY5Y cells

mAb	EC_{50} (nM)
7F5	0.26
11E1	0.05
17D11	0.07
19E12	0.05
29D7	0.47

SH-SY5Y cells were plated in 96-well plates at a density of 4×10^3 cells/well and incubated with 10 μm RA for 3 d to induce neuronal differentiation. Subsequently, cells were treated with varying concentrations of indicated mAbs, and the neurite outgrowth was measured using the Cellomics ArrayScan.

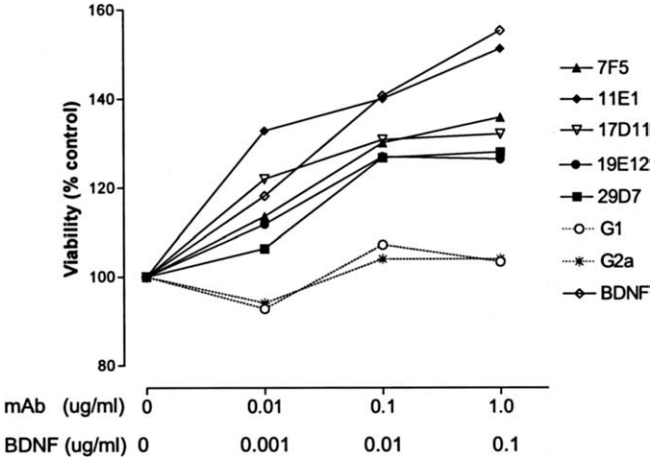

Figure 6. TrkB mAbs enhance the survival of differentiated SH-SY5Y cells. SH-SY5Y cells in 96-well plates were incubated with 10 μM all-trans-RA to induce neuronal differentiation. After 3 d, cells were incubated with serum-free media containing BDNF, TrkB mAbs, or isotype-matching control Abs (G1, mIgG1; G2a, mIgG2a), and cell viability was measured 2 d later using Cell Titer Cell Proliferation Assay. Data are presented as the percentage of increase viability over the vehicle-treated control.

levels of phospho-TrkB, phospho-MAPK, and phospho-Akt, as shown by immunoblot analyses (Fig. 7*G*), demonstrating the effectiveness of 29D7 toward activating neuronal TrkB receptors. We also tested the survival-promoting effects of 29D7 for cortical neurons. The survival of postnatal primary cortical neurons maintained in a B27-supplemented growth medium decreased in culture, resulting in a ~75% viability at 48 h compared with cultures grown in 5% serum-containing media. When varying concentrations of 29D7 were added to culture media, however, we observed dose-dependent rescue of cortical neurons, resulting in a significant increase in viability by 1 and 3 μg/ml 29D7 (124 ± 5 and 126 ± 1% viability vs vehicle control, respectively; $p < 0.05$; representative images of neurons shown in Fig. 7*H*).

Discussion

In this study, we describe selective and potent novel TrkB mAbs capable of activating TrkB receptors and thereby exerting neuroprotective and neurotrophic effects. These Abs bind functional TrkB receptors and are completely negative against TrkA or TrkC, confirming their specificity as TrkB receptor ligands. Analyses of signal transduction demonstrating TrkB phosphorylation, activation of MAPK and Akt, and CREB-mediated transcriptional activation provide evidence of bona fide TrkB-mediated responses after Ab treatment. Furthermore, unlike the endogenous agonists BDNF and NT-4, these TrkB Abs have no affinity toward p75NTR, as demonstrated by the lack of binding to p75NTR-overexpressing cells. Activation of this promiscuous low-affinity NTR negates the trophic effects of BDNF and even initiates cell death pathways and myelin inhibition (Bamji et al., 1998; Wong et al., 2002). Therefore, the Abs described here appear to possess a superior pharmacological profile that is not achieved by native ligands.

Most kinase growth factor receptors, including TrkB, are believed to be activated by agonist-induced dimerization, as evidenced by cross-linking studies (Jing et al., 1992). Although the details of this process remain to be elucidated, it has been postulated that agonist-induced receptor dimerization initiates structural transformation and stabilizes the tyrosine kinase activities associated with the intracellular domain of the receptors. The obligatory requirement of bivalency of ligands in receptor activation has been shown in a study by Clary et al. (1994), in which they demonstrated that monovalent anti-TrkA Fab fragments inhibited the biological effects of NGF, whereas bivalent TrkA IgGs were effective as NGF-mimetics in stimulating receptor activation, cell survival, and neurite outgrowth. More recently, Williams et al. (2005) reported that a cyclic peptide containing tandem repeats of a NT-4/TrkB-interacting motif could activate TrkB presumably by receptor dimerization, whereas a cyclic peptide with a single binding motif acted as a TrkB antagonist. Results from these studies suggest that monovalent agents behaved as competitive antagonists by simply occupying a ligand docking site and that receptor dimerization/oligomerization may be required to mimic the functional effects of ligands. Indeed, all

neurotrophins have been shown to form homodimers or heterodimers (Arakawa et al., 1994; Butte et al., 1998; Robinson et al., 1999), thus capable of bringing two Trk receptors together after binding. However, in one report, Saragovi and colleagues (Le-Sauteur et al., 1996) observed a functional agonism using monomeric Fab fragments of a human TrkA-specific mAb. In this study, treatment with Fab fragments increased phosphorylation of TrkA and protected cells from apoptotic death, suggesting that monomeric binding agents could induce activation of TrkA. Of interest is the observation that cross-linking Fabs using anti-Fab Abs could enhance survival responses. This suggests that receptor dimerization is of added benefit to cellular activity. Because our agonist TrkB Abs appear to activate the receptor by binding several discrete epitopes, it would be instructive to see if any of our Fab fragments are also functionally active. Furthermore, structural studies of Ab-bound TrkB using x-ray crystallography might reveal important conformational cues required for full receptor activation.

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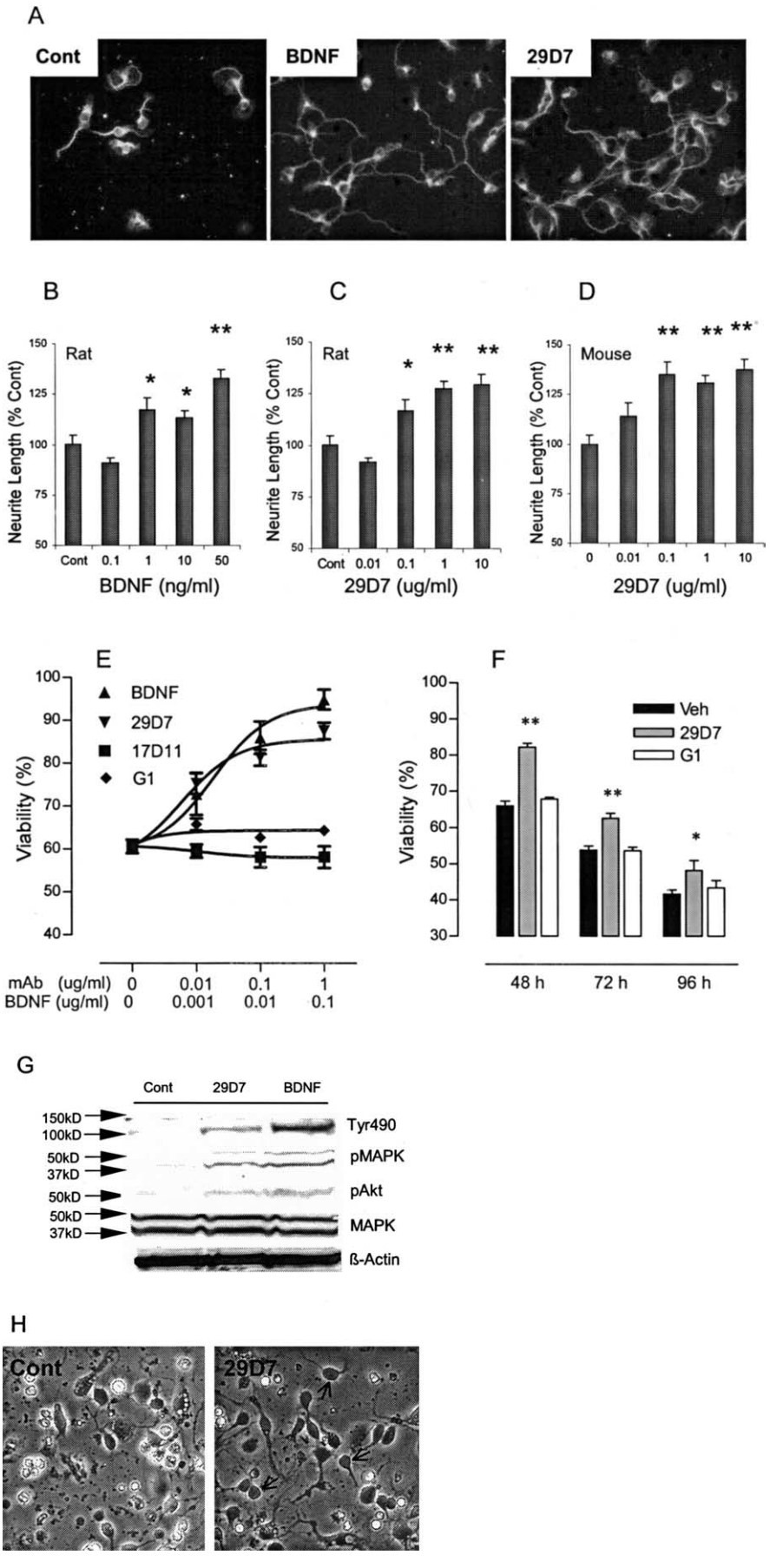

Figure 7. A TrkB mAb, 29D7, increases neurite outgrowth and neuronal survival in the primary neuronal cultures. ***A***, Representative images of TuJ1 immunostaining of rat cerebellar neurons treated with a vehicle, BDNF, (10 ng/ml), or 29D7 (0.1 μg/ml). ***B***, ***C***, Dissociated rat CGNs (postnatal day 7) were seeded onto 96-well plates in the presence of BDNF (***B***) or 29D7 (***C***) at indicated concentrations. ***D***, Effects of 29D7 on mouse CGNs. After a 24 h treatment, cells were fixed and neurite outgrowth was visualized by TuJ1 immunostaining. Data represent changes in the total neurite lengths after indicated treatments compared with a vehicle-treated control. $*p < 0.05$, $**p < 0.01$ compared with vehicle control determined by one-way ANOVA followed by Dunnett's *post hoc* test. ***E***, Rat CGNs were plated in a standard Neurobasal medium. The next day, serum and potassium were removed with or without the addition of BDNF or indicated Abs. Neuronal survival was measured 24 h after injury, and data are presented as the percentage of viability of uninjured neurons. All treatment concentrations of BDNF and 29D7 resulted in statistically significant enhancement of neuronal survival ($p < 0.05$ compared with the vehicle control, one-way ANOVA followed by Dunnett's *post hoc* test). ***F***, Rat CGNs treated with an indicated Ab (29D7 or control IgG1, 1 μg/ml) or a vehicle (Veh) were subject to serum/potassium deprivation as in ***E***, and the viability was measured at 48, 72, and 96 h after injury. $*p < 0.05$, $**p < 0.01$ compared with vehicle control (one-way ANOVA followed by Dunnett's test). ***G***, Rat CGNs on six-well plates were incubated with a vehicle, BDNF (100 ng/ml), or 29D7 (1 μg/ml) for 30 min, and an equal amount (20 μg) of proteins were resolved in the SDS-PAGE gel. Phosphorylation levels of TrkB, MAPK, and Akt kinases were probed using phospho-specific Abs, and the same samples were also probed for total MAPK protein and β-actin levels to verify equal loading. ***H***, Representative images of postnatal cortical neurons treated with or without 29D7 (1 μg/ml) for 48 h. The addition of 29D7 in culture medium increased the number of healthy neurons with intact processes (arrows). Cont, Control.

Our data from competition binding studies suggest that each of our five TrkB Abs recognizes a unique structural epitope of TrkB. We show that the binding of 7F5 or 11E1 to TrkB could be completely displaced by BDNF, suggesting that these Abs may recognize epitopes critical to TrkB–BDNF interactions. However, our data also suggest that 7F5 and 11E1 bind to unique epitopes, because even an excess amount of 11E1 could not completely compete with 7F5 binding onto TrkB. It is worth noting that 11E1 demonstrated the highest functional potency among our Abs in promoting neuritogenesis and cell survival in differentiated SH-SY5Y cells. This may suggest that the epitope recognized by 11E1 might be a critical domain regulating TrkB activation. Because the 11E1 epitope appears to be colocated with that of BDNF, it can be postulated that both 11E1 and BDNF activate TrkB through mechanisms involving common binding hot spots, similar to those illustrated in the crystal structures of a TrkB dimmer complexed with NT-4 (Banfield et al., 2001).

Compared with 7F5 and 11E1, Abs 17D11 and 19E12 showed different binding properties in that they inhibited BDNF binding only by 40–60%, even at saturating doses. ELISA avidities of these Abs to TrkB were similar to those of the BDNF-blocking Abs (7F5 and 11E1); hence, this cannot account for the differential efficacy in BDNF displacement. It is reasonable to postulate that the binding sites of these Abs only partially overlap with the BDNF docking sites, or that the binding of these Abs to TrkB induced an unfavorable conformational change of the BDNF docking site, allowing partial access of BDNF to TrkB in the presence of bound Ab. Again, despite similar BDNF-blocking activities, the precise binding epitopes of these two Abs appear not to be the same, as the binding of 17D11 could not be completely displaced by 19E12.

We also characterized another Ab, 29D7, that appears to possess the most distinct binding properties. 29D7 binds strongly to both hTrkB and mTrkB, and it recognizes an epitope that is completely distinct from the BDNF docking site. Not surprisingly, 29D7 does not compete with either BDNF or any of our other Abs. That both BDNF and 29D7 can bind TrkB concomitantly led us to the hypothesis that the addition of 29D7 would not negate functional activities of BDNF. Indeed, we confirmed this by showing that BDNF-mediated neurite outgrowth was not affected by the presence of 29D7 (our unpublished data). Therefore, this substantiates the existence of epitopes outside of the BDNF docking sites that can be targeted for TrkB activation without interfering with the physiological role of BDNF. As a number of studies have shown, upregulation of BDNF expression after traumatic injuries to the brain (Comelli et al., 1992; Dugich-Djordjevic et al., 1992; Yang et al., 1996) might represent part of a self-repair mechanism, and it is important that this effect should not be altered by administering reagents such as 29D7 that are non-BDNF competing. Intriguingly, we observed that the combination of BDNF with 29D7 was no more efficacious in promoting neurite outgrowth than treatment with either agent alone. One reason for this may be that there exists only a limited pool of receptors that can be dimerized and activated by agonist binding. However, this cannot fully explain our results because no synergy was observed by dual treatment even at submaximal concentration of either BDNF or 29D7.

Among the five Abs, 29D7 shows the highest affinity toward mTrkB (13 pM ELISA-binding ED_{50}). Therefore, it was important to know whether 29D7 would demonstrate functional activities toward endogenous TrkB. Using primary neuronal cultures, we showed that 29D7 could robustly induce receptor phosphorylation as well as activation of downstream signaling cascades. Furthermore, treatment with 29D7 increased the survival and growth of mouse and rat CGNs and rat cortical neurons, validating its functional effectiveness toward contextual neuronal systems. Surprisingly, 17D11, which also has a subnanomolar ELISA binding avidity to mTrkB, had no effects on either neurite growth or survival of mouse CGN. A lack of functional efficacy of 17D11 toward mTrkB is in contrast with its robust activity on hTrkB. This observation suggests that the 17D11 epitope on hTrkB is different from that on mTrkB and supports the notion that binding of a bivalent ligand itself may not be sufficient to induce receptor activation. Additional binding and structural analysis will be required to better understand this effect. To our knowledge, this is the first report that demonstrates isolation of human and rodent cross-reactive Abs using novel immunization techniques. By priming mice with antigens from both species, we successfully generated high-affinity cross-specific Abs. Because preclinical assessment of biological activity is critical to development of clinical candidates, this strategy might have a wide utility in isolating future therapeutic Abs.

In conclusion, we describe novel mAbs that selectively activate TrkB and thereby enhance neuronal survival and neurite outgrowth. Additional studies are in progress to address whether they have potential therapeutic use in ameliorating neurodegeneration associated with acute brain injury and/or promoting long-term repair.

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