An Agonistic mAb Directed to the TrkC Receptor Juxtamembrane Region Defines a Trophic Hot Spot and Interactions with p75 Coreceptors

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ABSTRACT: **The D5 domain of TrkC receptors is a docking site for Neurotrophin-3 (NT-3), but other domains may be relevant for function or harmonizing signals with p75^{NTR} coreceptors. We report a monoclonal antibody (mAb) 2B7 targeting the juxtamembrane domain of TrkC. mAb 2B7 binds to murine and human TrkC receptors and is a functional agonist that affords activation of TrkC, AKT, and MAPK. These signals result in cell survival but not in cellular differentiation. Monomeric 2B7 Fabs also affords cell survival. Binding of 2B7 mAb and 2B7 Fabs to TrkC are blocked by NT-3 in a dose-dependent manner but not by pro-NT-3. Expression of p75^{NTR} coreceptors on the cell surface block the binding and function of mAb 2B7, whereas NT-3 binding and function are enhanced. mAb 2B7 defines a previously unknown neurotrophin receptor functional hot spot; that exclusively generates survival signals; that can be activated by non-dimeric ligands; and potentially unmasks a site for p75-TrkC interactions.** © 2009 Wiley Periodicals, Inc. Develop Neurobiol 70: 150–164, 2010

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INTRODUCTION

Neurotrophin-3 (NT-3), brain-derived growth factor (BDNF), and nerve growth factor (NGF) are essential growth factors for the development and maintenance of the nervous system (Arevalo and Wu, 2006). The neurotrophins are stable homodimers (Butte et al., 1998) that bind to either or both two types of cell surface receptors termed p75^{NTR} and Trk.

Mature neurotrophins bind a selective Trk receptor with relatively high affinity (e.g., TrkA-NGF and TrkC-NT-3; K_d 10^{-11} M). The Trk receptor has tyrosine kinase catalytic activity that is associated with the survival and differentiation neurotrophic signals. Neurotrophin-induced Trk activity affords trophic (growth/survival) responses via MAPK and Akt, whereas PLC-γ and fibroblast growth factor receptor substrate-2 (FRS-2) activity are obligatory

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for differentiation (Kaplan and Stephens, 1994; Meakin et al., 1999).

A Trk receptor ectodomain termed D5 comprises the main neurotrophin binding site (Urfer et al., 1997; Wiesmann and de Vos, 2001; Zaccaro et al., 2001), and it is required for ligand-dependent receptor activation. Such receptor sites that define ligand binding and functional activation are termed "hot spots" (Saragovi and Zaccaro, 2002). Previously, we demonstrated that artificial ligands, such as antibodies, that bind to a receptor hot spot could be functionally active (Saragovi et al., 1999; Saragovi and Gehring, 2000; Saragovi and Zaccaro, 2002). Specifically, we reported an agonistic mAb 5C3 directed to the TrkA D5 domain (LeSauteur et al., 1996).

All mature neurotrophins also bind to $p75^{NTR}$ (Nykjaer et al., 2005), whereas the precursor proneurotrophins bind p75 exclusively (Nykjaer et al., 2004) and do not bind Trk receptors. The $p75^{NTR}$ can act either as proapoptotic or prosurvival depending on the ligand, developmental stage, or type of cell (Casaccia-Bonnefil et al., 1996; Bamji et al., 1998; Rabizadeh et al., 1999).

There is a reciprocal interplay between TrkA and p75 receptors that regulates signal cascades (Maliartchouk and Saragovi, 1997; Hapner et al., 1998; Maliartchouk et al., 2000; Ivanisevic et al., 2003) and ligand binding (Mahadeo et al., 1994). However, p75 regulates TrkC function differently than it does TrkA (Ivanisevic et al., 2003). For example p75 can be permissive of ligand-independent cellular differentiation through TrkC.

The mechanism by which p75 receptor affects Trk binding or function is still not fully understood but data show that p75 can unmask a cryptic "hot spot" of Trk receptors, supporting the notion of allosteric regulation (Zaccaro et al., 2001). In support of this, physical Trk-p75 interactions were reported in cells overexpressing these receptors (Bibel et al., 1999), but other data have suggested lack of TrkA-p75 interactions (Wehrman et al., 2007).

Here, we report the development of agonistic anti-TrkC mAbs as tools to explore whether novel hot spots could be defined outside the D5 domain of TrkC, to study whether and how p75 regulates TrkC function, and to evaluate the bioactivity of monovalent TrkC ligands.

MATERIALS AND METHODS

Cell Lines

Mouse SP2/0 myelomas, nnr5 cells (derived from rat PC12 pheochromocytoma) and, which express p75 but not Trk receptors, nnrr5 cells stably transfected with human *TrkC* cDNA (nnr5-TrkC), NIH-3T3 transfected with human *trkC* cDNA (NIH-TrkC cells) or human *trkA* cDNA (NIH-TrkA cells), and wild type NIH-3T3 cells were used. All cells were cultured in RPMI media supplemented with 5% fetal bovine serum (FBS) and antibiotics (Gibco). Stable transfectants were added the appropriate drug selection, and protein expression was routinely verified.

Coexpression of Full Length p75 Receptors

NIH-TrkC cells were stably transfected with full length rat p75 receptor with pcDNA3.1/Zeo(+) p75 construct. Stable transfectants were selected by treatment with Zeocin (200 μg/mL).

Antibodies

Rat anti-mouse IgG (αmIgG; Sigma, St. Louis, MO), anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY), anti-phospho-AKT (ser473) antibody (Cell Signaling), anti-phospho-MAPK (p42/44, thr202/tyr204) antibody (Cell Signaling), and fluoresceinated [fluorescin isothiocyanate (FITC)] goat anti-mouse IgG (FITC-GαmIgG) (Sigma, St. Louis, MO) and goat anti mouse Fab (GαmFab) antibodies were purchased commercially. mAb 5C3 was developed and grown in our laboratory (LeSauteur et al., 1996) and is an agonistic anti-TrkA mAb directed to the TrkA-D5/juxtamembrane domain. Rabbit antisera 203 that binds to all Trks was a gift of David Kaplan (Univ. of Toronto) and rabbit antisera to TrkC ectodomain protein was a gift of Lino Tessarollo (National Cancer Institute).

Peptide Immunogen

A peptide (ESTDNFILFDEVSPTPPI) spanning a sequence near the D5 domain of human TrkC was synthesized and was conjugated to KLH as carrier. The 18 amino acid ectodomain sequence is located at the linker region and ends 10 residues before the predicted transmembrane (TM) domain. The sequence matches perfectly and with no gaps most primates (e.g. chimpanzee), and has high homology with mouse and rat sequences (ESTDFFDFESDASPTPPI). The alignment for human/mouse/rat is ESTD-F–FD—+-SPTPPI.

mAb 2B7 Generation and Purification

All animal protocols were approved by McGill Animal Care Committee. Female Balb/c mice (8 weeks old) were immunized three times. Splenocytes were fused to SP2/0 myelomas, and hybridomas were screened by differential binding in an Enzyme-Linked Immunosorbent Assay (ELISA) using the original peptide immunogen conjugated to BSA. Specific binding data to native cell surface receptors were obtained using a fluorescent activated cell scanner

(FACScan) (Becton Dickinson, San Jose, CA) (see later). mAb 2B7 [IgG1(κ)] was identified by IsoStrip (Roche) and subcloned three times. mAb 2B7 was purified onto a Protein G-Sepharose column (Sigma). The binding and biochemical properties of purified mAb 2B7 were characterized by ELISA, FACScan, and SDS-PAGE.

Monomeric mAb 2B7 Fabs

mAb 2B7 was purified (8 mg/mL) as earlier and digested with 0.02 mg/mL papain (Gibco, Toronto, Ontario, Canada) for 6 h (LeSauteur et al., 1996). Fabs were repurified on Protein A-Sepharose and dialyzed against PBS. Products were characterized by SDS-PAGE under nonreducing conditions.

FACScan

Cells (2.5×10^5) in 0.1 mL of binding buffer [Hanks' Balanced Salt Solution 0.1% bovine serum albumin (BSA), and 0.1% NaN_3] were incubated with the indicated concentration of mAbs or Fabs for 20 min at 4°C, washed in binding buffer to remove excess primary antibody, and immunostained with FITC-GαmIgG secondary antibody for 20 min at 4°C. Cells were acquired and analyzed on a FACScan-BD Sciences using the Cell Quest program. As negative controls, no primary (background fluorescence) or irrelevant mouse IgG (Sigma) were used followed by secondary antibody. Specificity was gauged using various cells expressing different receptors.

Western Blot Analysis

Assays were performed as previously described (Maliartchouk and Saragovi, 1997). The activation of each protein (Trk, Akt, and MAPK) was studied after treatment of live cells with different concentrations of ligands mAb 2B7, mAb 2B7 Fabs, NT-3 for 12 min at 37°C; cells were solubilized and protein concentrations were determined with Bio-Rad Detergent Compatible Protein Assay (Bio-Rad). Western blot analysis was performed with the indicated reagents. Blots were visualized using the enhanced chemiluminiscence system (PerkinElmer Life Sciences). Reblotting the membranes with antiserum directed to total Trk (203 serum from Dr. David Kaplan) or anti-actin antibody (Sigma) confirmed equal protein loading. Quantification of Western blots was done by densitometric analysis relative to total protein levels. Quantification data are presented as percent relative to optimal (10 n*M* NT-3) as 100%. Statistical analysis were performed by two-tailed *t*-tests; statistical significance ($p \leq 0.05$) is indicated by an asterisk (*).

Binding Inhibition Assays

mAb 2B7 and mAb 5C3 were labeled with biotin (Pierce). Competition of mAb 2B7 binding to NIH-TrkC or NIH-TrkC+p75 cells was tested with NT-3 or NGF as irrelevant control. The binding assays were first optimized to quantify saturation. Cells were first incubated with various concentrations of the test inhibitor (20 min at 4°C) followed by saturating ($\sim$67 n*M*) of mAb 2B7-biotin, mAb 5C3-biotin as irrelevant primary, or negative control mouse IgG for another 20 min at 4°C. Then, FITC-goatαmIgG or FITC-avidin was added as secondary reagent. After washing, cells were analyzed by FACScan as previously described. The conditions used (4°C and Na azide in the buffer) did not allow internalization.

Proliferation/Survival Assays

NIH-TrkC or NIH-TrkC+p75 cells (7,500 cells/well) in serum-free media (SFM) (PFHM-II; Gibco) supplemented with 0.2% BSA were added to 96-well plates (Falcon, Lincoln Park, NJ) containing NT-3, mAb 2B7, mAb 2B7 Fabs, negative control mouse IgG, or serum (final 5% FBS, normal culture conditions). Where indicated, mAb 2B7 Fabs were crosslinked with goat anti-mouse Fab (Gαm Fab). Wells containing all culture conditions but no cells were used as blanks. The growth/survival profile of the cells was quantified using the tetrazolium salt reagent 4-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) 48–72 h after plating. Optical density readings of MTT were done in a Benchmark Plus microplate Spectrophotometer (BioRad) at 595 nm with blanks subtracted. For the cells used in this article, we have validated the MTT method for measuring cell viability (Maliartchouk and Saragovi, 1997; Maliartchouk et al., 2000; Ivanisevic et al., 2003). Almost all the cells die by apoptosis within 72 h. In this assay, "partial survival" could also mean "slower death," but given that Trkc-pTyr is activated we favor the view of partial survival.

Differentiation Assays in Cell Lines

nnr5 cells stably transfected with *TrkC* cDNA (nnr5-TrkC) were plated on cover slips with full media in 24-well plates. Twenty four hours after plating, the indicated treatments were added for an additional 48–72 h of culture. Cellular differentiation was gauged by immunocytochemistry after cover slips were fixed and stained with MAP-2 antibody (Chemicon) followed by goat anti-rabbit Cy3 (Jackson Immunochemicals) and analyzed as described earlier (Ivanisevic et al., 2003).

Dendritic Development Assays in Hippocampal Neurons

Primary cultures of hippocampal neurons were prepared from rats (E18) as previously described (Kaech and

Banker, 2006). Neurons were plated on poly-L-lysine coated coverslips at low density ($\sim$6000 neurons/cm^2) in minimum essential medium containing 10% horse serum, 0.6% glucose, 2 m*M* glutamine, and antibiotics. After 5–8 h, medium was replaced for Neurobasal media (GIBCO, Invitrogen, USA) containing 1 m*M* glutamine, B-27 supplement, and antibiotics. These neuronal cultures do not express detectable p75 (data not shown, see (Bronfman et al., 2007)). This is consistent with this stage for neurons *in vivo*, where p75 is undetectable by immunohistochemistry in the hippocampal formation of the adult rat brain (Lee et al., 1998). One day after plating, neurons were treated with saturating concentrations of neurotrophins (NT-3, BDNF, 6.7 n*M*) or 2B7 (100 n*M*). After 7 days of treatment, neurons were fixed 15 min with 3% paraformaldehide, 4% sucrose in PBS. For immunostaining, fixed neurons were incubated with glicyne 0.15 *M*, pH 7.4, for 10 min and then washed. Neurons are then permeabilized with 0.2% saponin, nonspecific binding was blocked with 3% BSA, and immunostaining was done with mAb MAP2 (Chemicon, Millipore), followed by incubation with anti-Mouse Alexa 555 secondary antibody (Molecular Probes).

Image Analysis and Quantification

Z-series of individual immunostained neurons was acquired with a Zeiss LSM Pascal 5 (Carl Zeiss, USA) connected to an inverted microscope (Axiovert) with a 63$\times$ objective. Z-series of each neuron were integrated in a single image for morphometrical analysis of the complete dendritic arbor. Quantitative analysis of dendritic arborization was performed using ImageJ software (NIH, USA) as previously described by Sholl (Sholl, 1953; Rema et al., 2008). For each neuron, concentric circles spaced 10 μm apart starting from the center of cell body was traced. The number of dendrites that intersect each circle was counted and plotted as a function of distance from the soma. The total length of neurites and branch length per branch order was analyzed using the "ImageJ" plugin called "NeuronJ". Neurites were traced manually and labeled as primary (originated directly from the soma), secondary (branching from a primary), and tertiary (branching from a secondary). As the MAP2 antibody labels all neurites, it is not possible to accurately assign axons and dendrite nomenclature. Instead, the program uses an order of primary-secondary-tertiary branches to perform the calculations. Once branch order nomenclatures were assigned, the neurite tracings appeared color coded by type and a text file containing neurite total length and branch length per branch order measurement data were generated. The average of the total length of neurites and average of branch length per branch order were calculated, and *t*-test was applied for statistical analysis ($p \leqslant 0.05$ considered significant).

RESULTS

Generation and Initial Screening of mAb 2B7 Binding

Linear peptide NH_2-ESTDNFILFDEVSPTPPI-COOH was conjugated through the N-termini to KLH and was used to immunize mice. After fusion of splenocytes with SP2 myeloma cells, culture supernatant from hybridomas was screened by ELISA using either the immunizing peptide conjugated to BSA or free peptide immobilized on the ELISA plate. Several independent wells with hybridoma cells producing antibodies with selectivity to the immunizing peptide were identified, and they were subcloned three times by limiting dilution. mAb 2B7 was chosen for further work.

Characterization of mAb 2B7 Binding

To assess mAb 2B7 specificity for cell surface TrkC, cells expressing or lacking TrkC were screened for differential binding by FACScan.

mAb 2B7 binds strongly to NIH-TrkC transfectants [Fig. 1(C)] and nnr5-TrkC transfectants [Fig. 1(F)]. In controls, it does not bind to wild type NIH-3T3 cells [Fig. 1(A)], NIH-TrkA transfectants [Fig. 1(B)], or wild type nnr5 cells [Fig. 1(E)] above mIgG background control. In additional assays, mAb 2B7 does not bind to SY5Y cells transfected with human TrkB cDNA (data not shown). Binding of mAb 2B7 to nonpermeabilized cells indicates that it recognizes the extracellular domain of TrkC. The specific epitope on TrkC is located between the transmembrane and the D5 domain. Therefore, mAb 2B7 binds selectively to native TrkC on the cell surface near the juxtamembrane region, and it does not bind to p75, TrkB, or TrkA.

The concentration of mAb 2B7 and mAb 2B7 Fab required for saturation of TrkC [Fig. 1(I)] is $\sim$65 n*M* mAb 2B7 and $\sim$75 n*M* 2B7 Fab. The slight difference in fluorescent intensity at saturation with intact mAb versus Fabs is because of the use of different fluorescinated secondary reagents.

Western blot analysis with mAb 2B7 reveals a band at Mr 145 kDa (p145) for lysates from NIH-TrkC but no bands for lysates of control wild type NIH-3T3 cells [Fig. 1(J)] or for NIH-TrkA cells (data not shown). mAb 2B7 is effective in western blot analysis only when samples were prepared under nonreducing conditions, which suggest the influence of a disulfide bond.

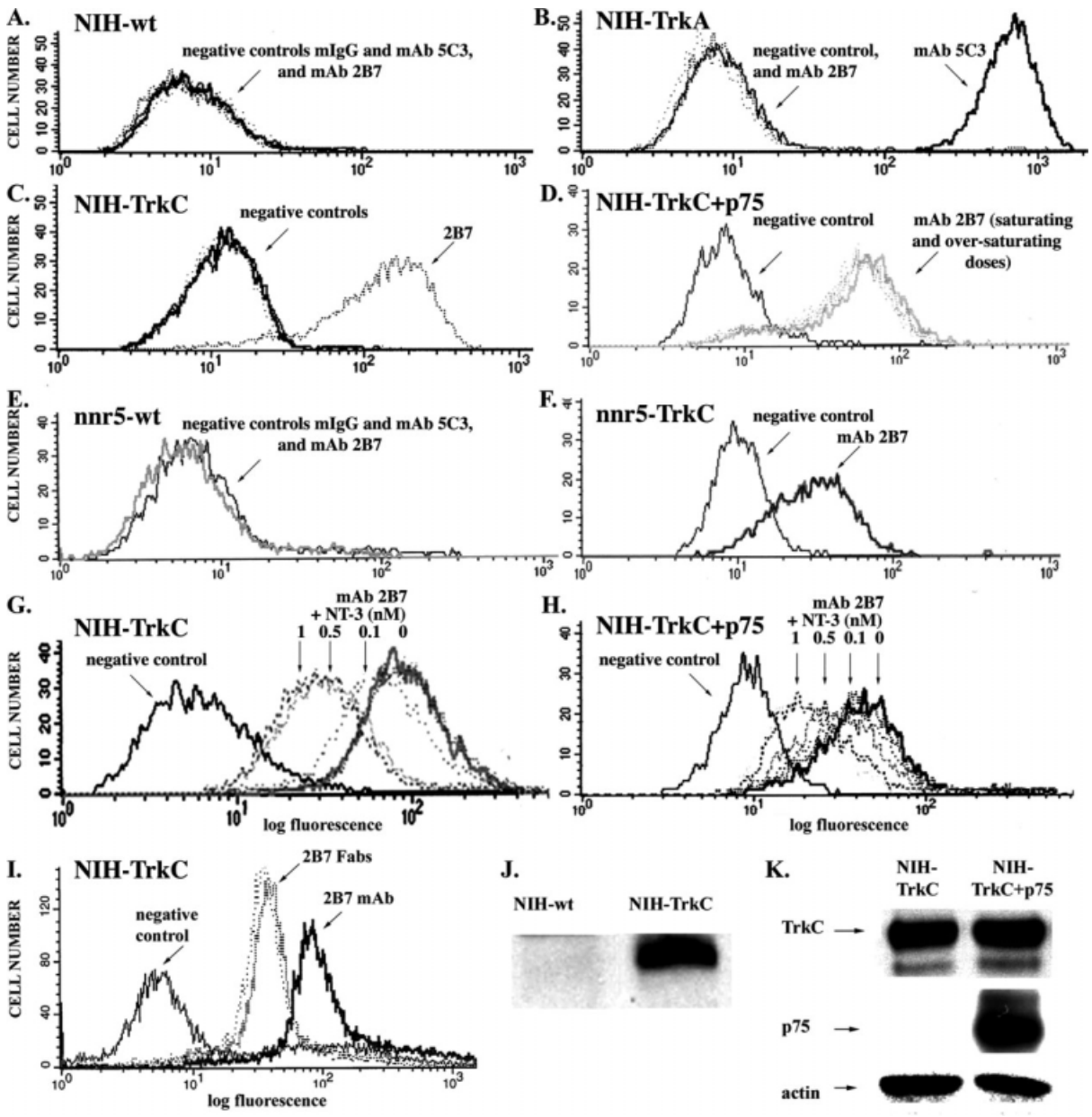

Figure 1 mAb 2B7 binds selectively to TrkC receptor expressing cells. All data are representative of at least three independent assays. A–F: FACScan binding assays with mAb 2B7. The indicated cells were incubated with saturating concentration of mAb 2B7 ($\sim$65 n*M* or higher). G, H: Dose-dependent competition of mAb 2B7 binding by NT-3. NIH-TrkC or NIH-TrkC+p75 cells were studied. Note reduced mAb 2B7 immunofluoresence in NIH-TrkC+p75 cells although they express the same levels of TrkC (see panel K). I: Comparable FACScan saturability profile in NIH TrkC cells for mAb 2B7 and 2B7 Fabs. J: 2B7 recognizes TrkC in Western blots, under nonreducing conditions. K: Total levels of TrkC receptor are similar in NIH-TrkC and NIH-TrkC+p75 cells. NIH-TrkC+p75 cells express high levels of p75. The doublet seen for TrkC may be differentially glycosylated receptor (Watson et al., 1999).

Ligand Competition Studies

FACScan analysis demonstrated that NT-3 blocks, in a dose-dependent manner, mAb 2B7 binding sites. NT-3 at 1 n*M* blocks $\sim$40% of the mAb 2B7 binding sites in NIH-TrkC [Fig. 1(G)] and in NIH-TrkC+p75 cells [Fig. 1(H); see Table 1 for a summary]. Thus,

Table 1 NT-3 Competes 2B7 Binding

Fluorescent Antibody	Added NT-3 Competitor	Mean Channel Fluorescence		% Inhibition of 2B7 Binding	
		TrkC Cells	TrkC + p75 Cells	TrkC Cells	TrkC + p75 Cells
2B7	0 n*M*	95	56		
2B7	0.1 n*M*	87	42	14 ± 4	30 ± 2
2B7	1.0 n*M*	63	33	43 ± 8	49 ± 5
mIgG control	–	11	10		

Summary of FACScan data from Figure 1G and 1H. Raw mean channel fluorescence (MCF) for a single experiment is shown, and mAb 2B7 inhibition of binding by NT-3 are presented as % inhibition ± sem, $n = 3$ independent experiments. Mouse IgG is used as background control (no binding). mAb 2B7 is used at saturating concentrations.

mAb 2B7 binds to a receptor hot spot topologically related to the NT-3 binding site.

Coexpression of p75^{NTR} Reduces the 2B7 mAb Binding Sites on TrkC, an Effect That Requires the Extracellular Domain of p75^{NTR}

Coexpression of full length p75^{NTR} in NIH-TrkC cells reduces the cell surface 2B7 binding sites by ~50–60% in quantitative FACScan assays. This reduction was observed in all 12 NIH-TrkC+p75 clones that were independently isolated [Fig. 1(D), also see Table 1]. Higher concentrations of mAb 2B7 do not overcome the reduction in binding sites elicited by expression of p75.

Quantitative western blot analyses of three NIH-TrkC+p75 clones, using mAb 2B7, demonstrated levels of total TrkC comparable to those in parental NIH-TrkC cells [Fig. 1(K)], and similar data were obtained using anti-TrkC rabbit serum (data not shown). These data indicate that expression of p75 does not reduce expression of TrkC. Rather, expression of p75 may induce conformational changes or steric hindrance at or near the mAb 2B7 epitope on TrkC. This would be suggestive of physical or allosteric p75•TrkC interactions that prevent mAb from binding to TrkC. This suggestion is further supported by the observed inverse correlation between p75 levels and the level of blocking of mAb 2B7 binding to TrkC. In different clones, low expression of p75 reduces 2B7 binding weakly, whereas high expression of p75 reduces 2B7 binding strongly (data not shown).

To determine which domain of p75 was relevant to block the 2B7 mAb binding sites on TrkC, we transfected a p75 construct expressing the TM domain and intracellular domain (ICD) but which had the ectodomain (ECD) deleted. High expression of the deletion p75 mutant was verified through an engineered tag. The expressed p75-TM-ICD is not sufficient to block mAb 2B7 binding to TrkC (data not shown). Thus, we conclude that the p75 ECD is required for blocking mAb 2B7 binding to TrkC.

p75^{NTR} Blocking of 2B7 mAb Is Not Regulated by p75 Ligands

Because the p75 blocking of 2B7 mAb binding requires the p75-ECD, we tested whether selective ligands of p75 could release the hindrance to 2B7•TrkC interactions.In NIH-TrkC+p75 cells, NGF, BDNF, and pro-NT-3 (Alomone Labs) were added first to engage p75 because in this paradigm they act as p75-selective ligands. Then, cells were analyzed in quantitative FACScan assays with mAb 2B7. None of the p75 ligands afford an increase or a decrease in 2B7 binding (data not shown). The data suggest that whether or not it is liganded, p75 can reduce 2B7•TrkC binding.

As an interesting side point, pro-NT-3 does not reduce the mAb 2B7 binding sites on NIH-TrkC cells either (data not shown), whereas mature NT-3 does [Fig. 1(G)]. These data indicate that pro-NT-3 does not bind to this particular region of TrkC receptors.

Agonism by mAb 2B7 and Monomeric mAb 2B7 Fabs

Biochemical assays (see Fig. 2), survival assays (see Fig. 3), and differentiation assays (see Fig. 4) were undertaken to determine if mAb 2B7 has NT-3-like agonistic activity.

Phosphorylation of TrkC, AKT, and MAPK were studied in lysates from cells that had been exposed for 12′ to ligands or controls (see Fig. 2). NT-3, mAb 2B7, and 2B7 Fabs (with or without crosslinking using GαmFab antibodies) afford significant tyrosine phosphorylation of TrkC (p-TrkC) over basal levels in untreated cells [Fig. 2(A)].

NT-3, mAb 2B7, and 2B7 Fabs also activate downstream signaling proteins MAPK (~twofold

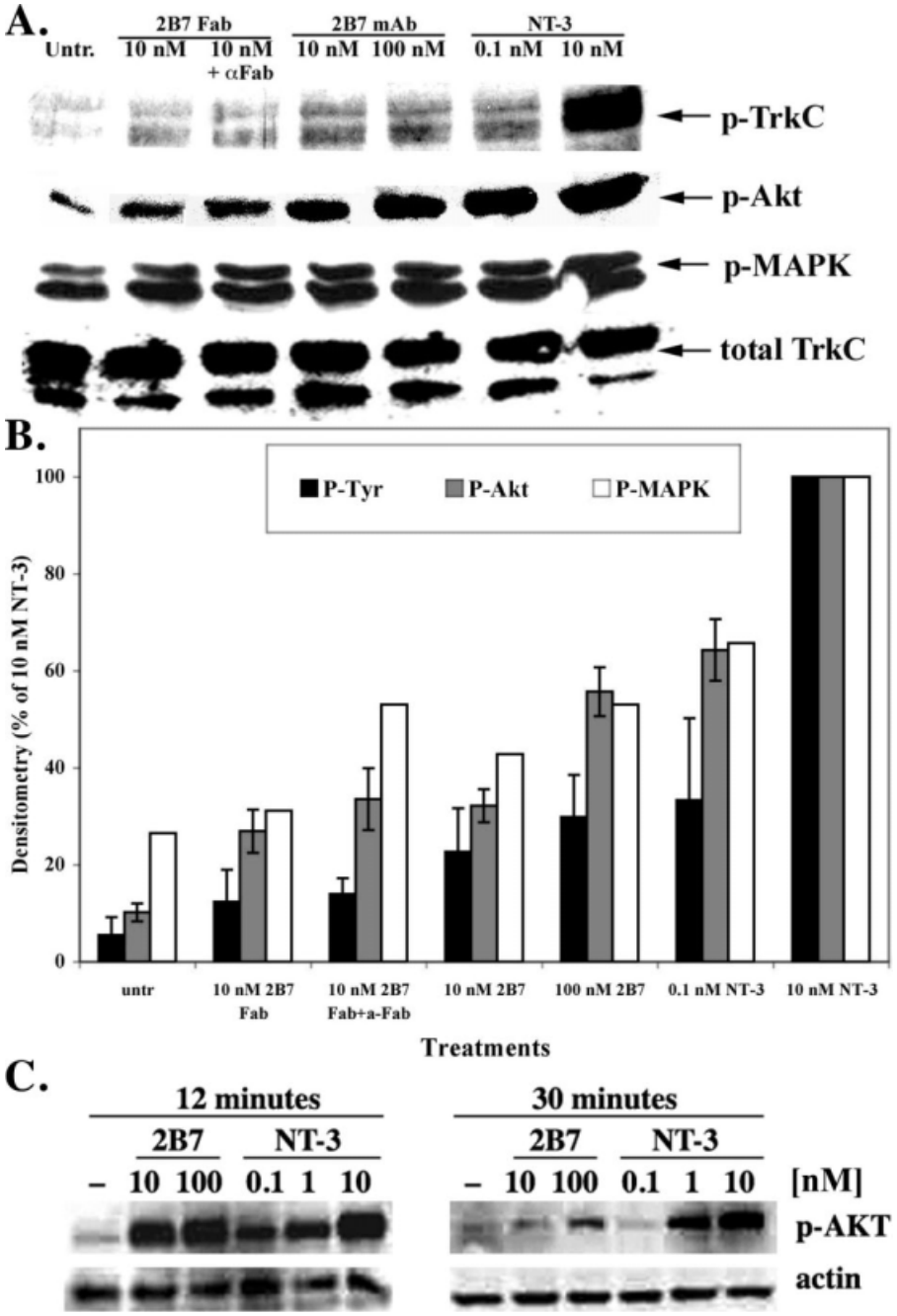

Figure 2 TrkC, Akt, and MAPK activation are induced by mAb 2B7 or mAb 2B7 Fabs. NIH TrkC cells were treated with the indicated ligands for 12′ and cell lysates were analyzed by Western blots. A: anti-P-Tyr, anti-P-Akt, anti-P-MAPK, and total Trk (anti-sera 203, from Dr. David Kaplan). Representative experiment is shown. B: Summary of data quantification by densitometry standardized to total Trk and presented as % relative to 10 n*M* NT-3. Anti-P-Tyr $n = 3$; anti-p-Akt $n = 3$; anti-p-MAPK $n = 2$. C: anti-p-Akt blots as in (A) NIH TrkC cells treated with the indicated ligands for 12′ or 30′.

over baseline) and AKT (∼fivefold over baseline) [Fig. 2(A)]. The quantification of phosphorylated proteins after 12′ of activation, relative to total Trk protein loaded, are presented as % of optimal NT-3 (10 n*M*, 100% efficacy) [Fig. 2(B)]. p-TrkC is induced by 0.1 n*M* NT-3 or by 10 n*M* 2B7 with ∼30% efficacy and by 2B7 Fabs (or 2B7 Fabs cross-linked with GαmFabs) with ∼15% efficacy. p-Akt

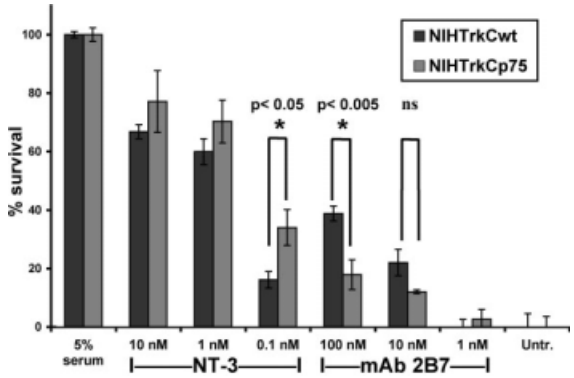

Figure 3 Coexpression of p75 with TrkC hinders the trophic protection of mAb2B7 The survival of NIH-TrkC or NIH-TrkC +p75 cells was tested in MTT assays after culture in SFM supplemented with the indicated ligands or controls (5% serum = 100%, untreated = 0%), $n = 4$ each assay. Data are representative from 3 independent experiments. * indicates statistical significance. ns indicates not statistically significant.

and p-MAPK are induced by 0.1 n*M* NT-3 and 10 n*M* 2B7 with ~60% efficacy and by 2B7 Fabs (or 2B7 Fabs crosslinked with GαmFabs) with ~30% efficacy. In cellular controls studying NIH-TrkA cells, there is no increase in p-TrkA, p-AKT, or p-MAPK after treatment with 2B7 mAb or 2B7 Fabs (data not shown).

A longer time-course study of p-AKT (12′ and 30′ of ligand treatment) showed that activation by NT-3 was sustained, whereas activation by 2B7 was less efficient long term. At the 30 min point p-AKT by NT-3 remains high ~70% relative to that seen at 12′, whereas p-AKT by 2B7 is significantly reduced and is <20% relative to that seen at 12′ [Fig. 2(C)]. Therefore, activation by mAb 2B7 is transient compared with that of NT-3.

These ligands were then tested for their ability to protect cells from death induced by culture in SFM (see Fig. 3). This cellular death is known to be apoptotic. In MTT assays, NT-3, mAb 2B7, and monovalent 2B7 Fabs significantly delay the death of NIH-TrkC cells in a dose-dependent manner. Compared versus optimal NT-3, mAb 2B7 has a maximal efficacy of ~45%. Monomeric 2B7 Fabs have a maximal efficacy of ~35% (data not shown). These data correlate with induction of pAKT, which is known to be involved mediation of trophic support.

Similar MTT assays compared the effects of TrkC agonists on NIH-TrkC cells versus NIH-TrkC+p75 cells (see Fig. 3). The purpose was to test the effect of p75 expression on 2B7 agonistic activity. Because these two cell lines have different survival profiles in response to NT-3, the data here are standardized to normal serum growth conditions (100%). The death of NIH-TrkC and NIH-TrkC+p75 cells are both reduced by 10 n*M* NT-3 to a comparable degree ~65–70%. As expected, expression of full-length rat p75 in NIH-TrkC cells significantly enhances the efficacy of 0.1 n*M* NT-3 (35% survival in NIH-TrkC+p75 versus 20% in NIH-TrkC). In contrast, p75 expression significantly reduces the efficacy of mAb 2B7 (20% survival in NIH-TrkC+p75 cells versus 45% in NIH-TrkC).

Thus, expression of p75 improves the binding and the function of NT-3 but reduces the binding and the function of mAb 2B7. These data correlate with a reduction of mAb 2B7 binding when p75 is coexpressed.

Effect of p75 Ligands on 2B7 Agonistic Activity

We have previously shown that p75 negatively regulates the efficacy of selective TrkA agonists such as mAb 5C3 (LeSauteur et al., 1996). In this scenario, p75 ligands such as anti-p75 mAb MC192 neutralize the negative regulation of p75 and thus allow full TrkA activation (Maliartchouk and Saragovi, 1997). We therefore performed survival assays with mAb 2B7 ± engagement of p75 with NGF, BDNF, or anti-p75 mAb MC192.

The ligands are p75-selective in NIH-TrkC+p75 cells and do not enhance or reduce the survival-promoting signals of mAb 2B7 (data not shown). Thus, the negative regulation of p75 upon mAb 2B7 survival function is not affected by p75-ligands. These data are consistent with our earlier data showing that p75-ligands do not reverse the block to mAb 2B7 binding and with a report that p75•TrkC functional interactions differ from p75•TrkA functional interactions (Ivanisevic et al., 2003).

Effect of mAb 2B7 on the Differentiation of Cell Lines

We next tested neurite outgrowth in response to mAb 2B7 [Fig. 4(A), data summarized in Fig. 4(B)]. Treatment with mAb 2B7 does not induce the differentiation of nnr5-TrkC cells. This was puzzling because mAb 2B7 binds to the cell surface of nnr5-TrkC cells [Fig. 1(F)]. In positive control assays, nnr5-TrkC cells differentiate in response to NT-3, in a dose-dependent manner. NT-3 increases the percent of cells bearing >2 axons with axonal length >2 cell bodies. In negative control assays, treatment with mIgG or 10 n*M* NGF do not differentiate nnr5-TrkC cells.

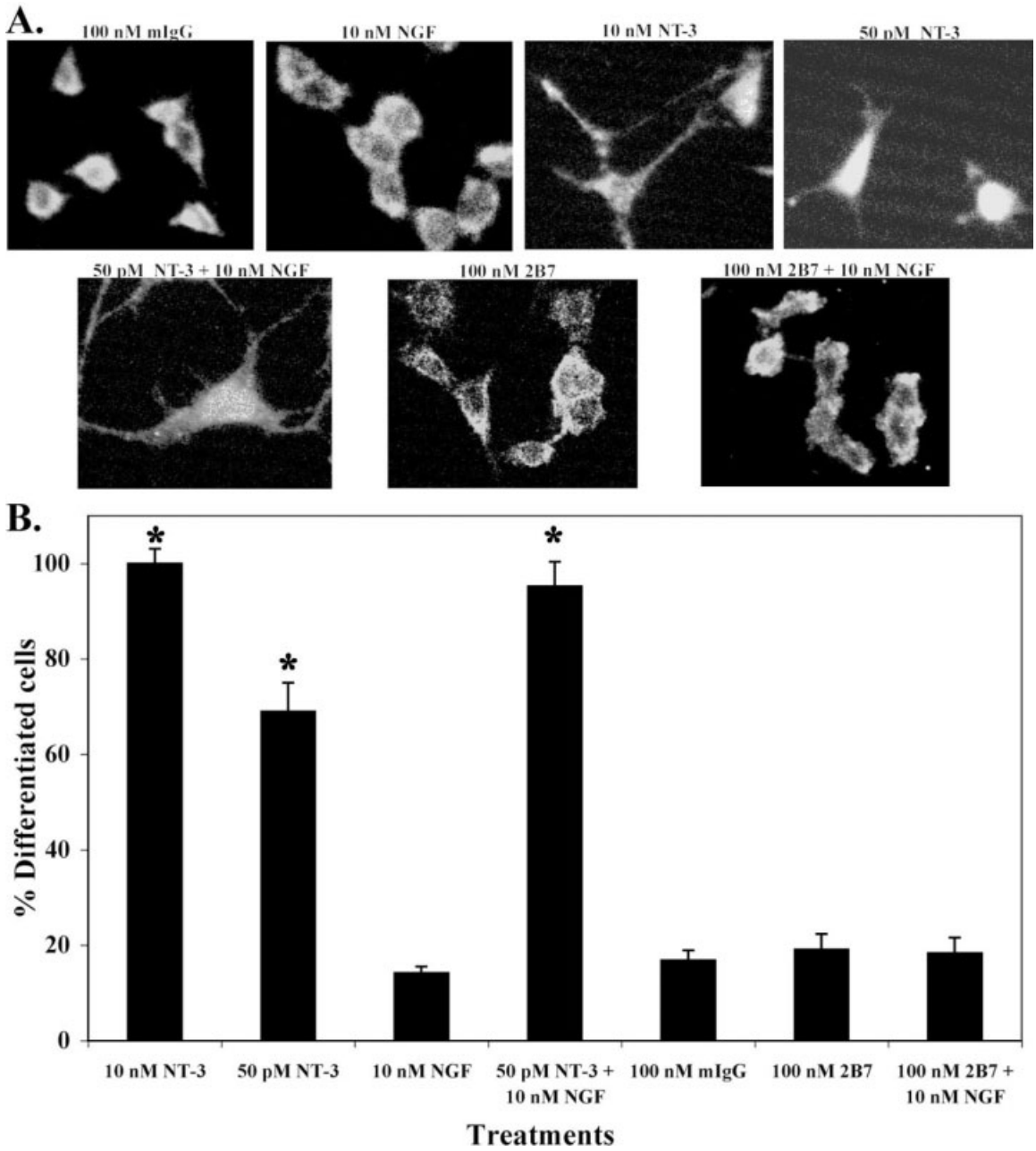

Figure 4 mAb 2B7 does not induce differentiation of nnr5 TrkC cells. A: Representative pictures of the differentiation of nnr5-TrkC cells in response to treatment with the indicated ligand for 48–72 h, and cells were then fixed and immunostained with MAP-2 antibody (Chemicon) followed by goat anti-rabbit Cy3 (Jackson Immunochemicals) and analyzed as described (Ivanisevic et al., 2003). B: Quantitative summary (±SD) of 3 independent experiments. Cells were plated with indicated treatments or controls, and differentiation was scored as % of cells with neurites (> 2 cell body long). * indicates statistical significance relative to 100 n*M* mIgG, $p \leqslant 0.05$. A total of 50 p*M* NT-3 affords significantly lower cellular differentiation than 10 n*M* NT-3. A combination of 50 p*M* NT-3 + 10 n*M* NGF (as a p75 ligand) affords significantly higher cellular differentiation than each ligand alone and achieves levels comparable to 10 n*M* NT-3.

Because p75 can regulate TrkC-mediated signals, including cellular differentiation (Ivanisevic et al., 2003), we tested whether mAb 2B7 in combination with selective p75 ligands may afford cellular differentiation. mAb 2B7 combined with the p75-selective ligands NGF (see Fig. 4) or BDNF (data not shown)

do not stimulate differentiation. In contrast, in positive controls, a suboptimal concentration of 50 p*M* NT-3 in combination with NGF as a p75-selective ligand increases cellular differentiation. This control combination achieves levels comparable to optimal 10 n*M* NT-3, as reported previously (Ivanisevic et al., 2003).

Thus, mAb 2B7 activates TrkC but does not have intrinsic neuritogenic activity in the nnr5-TrkC cell line, and the use of p75 ligands do not potentiate neurogenesis either.

Effect of BDNF, NT-3, and mAb 2B7 on Dendritic Arborization in Primary Neuronal Cultures

Primary cultures of E18 rat hippocampal neurons were prepared (Kaech and Banker, 2006) and plated at low density ($\sim$6000 neurons/cm^2) as described in Neurobasal/B27. Under these conditions, expression of p75 is barely detectable by western blotting and is undetectable by immunohistochemistry (Bronfman et al., 2007). This is consistent with this stage of development for neurons *in vivo*, where p75 is undetectable by immunohistochemistry in the hippocampal formation of the adult rat brain (Lee et al., 1998). Neuronal cultures were treated with saturating concentrations of NT-3, BDNF (6.7 n*M*), or 2B7 (100 n*M*), and the morphology of the dendritic arbor was analyzed after 7 days treatment as described in the Methods.

The data of the morphology of the dendritic arbor are illustrated in Figure 5. The histogram on Figure 5(A) represents the Sholl analysis of dendritic intersections as % of untreated control cells. Both, NT-3 and BDNF have a significant but differential effect on the morphology of the dendritic arbor. Both NT-3 and BDNF treatment results in a higher number of primary dendrites (projections from the cell body of the neuron) and an increase in dendritic branching. However, NT-3 resulted in longer dendrites compared with BDNF. Compared to NT-3 or BDNF the mAb 2B7 did not have any effect on the development of the dendritic arborization of hippocampal neurons, suggesting that the mAb is not able to trigger morphological differentiation as NT-3.

The average of the total length of neurites and average of branch length per branch order was further assessed using NeuronJ [Fig. 5(B)]. Both BDNF and NT-3 significantly increased the total length of neurites compared with control-untreated cells ($p < 0.05$, $p < 0.01$, respectively). Interestingly, NT-3 treatment resulted in longer neurites compared with BDNF

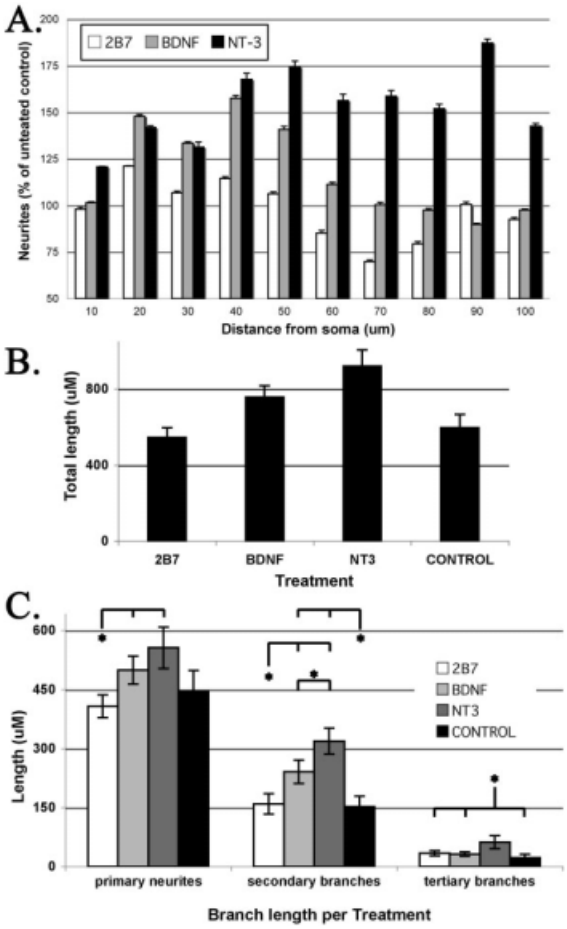

Figure 5 mAb 2B7 does not induce differentiation of primary neuronal cultures. Hippocampal neurons were plated with indicated treatments, and differentiation was scored as % of control cells. A: Sholl analysis of dendritic intersections as a % of untreated control cells. NT-3 and BDNF have a significant effect on the morphology of the dendritic arbor, with a higher number of primary dendrites and branching. NT-3 has longer dendrites compared with BDNF, whereas mAb 2B7 did not have any effect on the development of the dendritic arborization. B: The average of the total length of neurites and average of branch length per branch order was further assessed using NeuronJ. BDNF and NT-3 significantly increased the total length of neurites compared with control-untreated cells, but NT-3 treatment was more effective. The mAb 2B7 did not have any effect on the growth of neurites compared to control. All comparisons are statistically significant ($p < 0.05$) except control versus 2B7 treatment. C: Analysis of the branch length per branch order. mAb 2B7 did not cause any change in the elongation of neurites. NT-3 increases the length of both secondary ($p < 0.001$) and tertiary order branches ($p < 0.05$), whereas BDNF increases the length of secondary order branches ($p < 0.01$). Asterisk indicates $p < 0.05$. This analysis is consistent with the Sholl analysis in panel A.

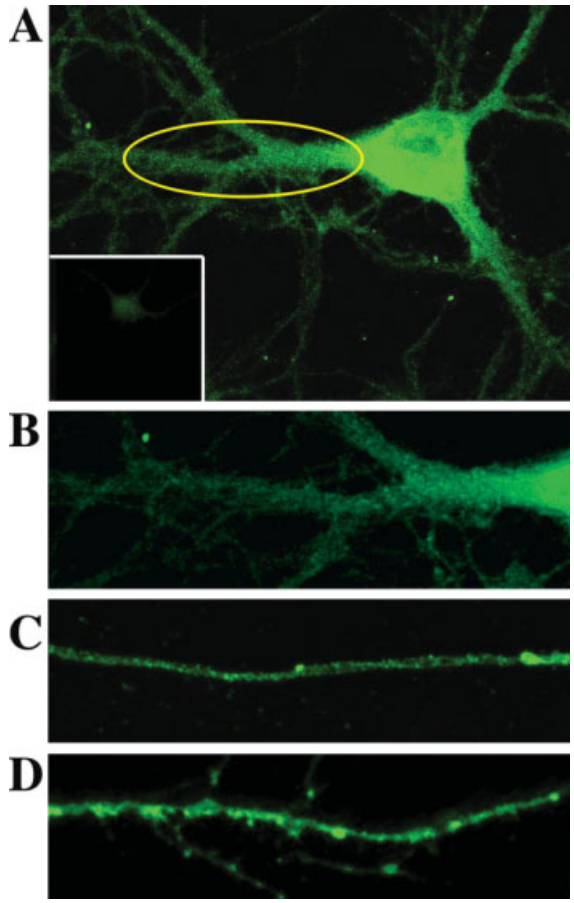

Figure 6 mAb 2B7 imunostaining of primary neuronal cultures. Representative pictures of E18 primary hipoccampal neurons immunostained with mAb 2B7. A–C: Confocal images. D: epifluoresence. Control immunostaining with no 2B7 primary results in no detectable fluorescent signal (inset square, panel A). The image in panel B is a magnification of the area in yellow ellipse from panel A.

($p < 0.05$). The mAb 2B7 did not have any effect on the growth of neurites compared to control.

A further detailed analysis of the branch length per branch order [Fig. 5(C)] reveals that, compared to control, BDNF increases the length of secondary order neurites ($p < 0.01$), whereas NT-3 increases the length of both secondary ($p < 0.001$) and tertiary order neurites ($p < 0.05$). NT-3 had a bigger effect on the length of secondary and tertiary branches compared to BDNF ($p < 0.05$), indicating that NT-3 results in longer neurites, as we have indicated by Sholl analysis.

Representative confocal immunofluorescence and epifluorescent pictures of neuronal cultures immunostained with mAb 2B7 are shown (see Fig. 6). The neuronal cultures look healthy, even if they are not supplemented with growth factor or antibody. Low magnification shows widespread and intense immunostaining of soma and dendrites [Fig. 6(A,B)]. High magnification shows punctate immunostaining in the axons, that appear to be vesicular [Fig. 6(C,D)].

Thus, the mAb 2B7 did not induce any change in the elongation of neurites in hippocampal neurons. These data are consistent with the results using cell lines and suggest that 2B7 can support cell survival but does not have any effect on the differentiation process.

DISCUSSION

The mAb 2B7 reported in this study specifically binds to the TrkC ectodomain, at the juxtamembrane linker region. It is useful for FACScan, immunofluorescence analysis, immunoprecipitation, Western blot analysis, and immunocytochemistry of human TrkC and rat TrkC (this report) and murine TrkC (Esteban et al., 2006).

The epitope of mAb 2B7 defines a previously unknown "hot spot" of TrkC, between the second immunoglobulin domain (D5) and the TM domain. Competition studies between NT-3 and mAb 2B7, shown here, indicate topological closeness at their binding sites such that at least steric inhibition can occur. On the other hand, crystallographic analyses have indicated that the juxtamembrane region is not required for NT-3 binding (Urfer et al., 1998; Ultsch et al., 1999; Wiesmann and de Vos, 2001). However, this region has generally been excluded from analyses as it is truncated in engineered proteins used for crystallography. Thus, while it may not be absolutely required, its relevance can not be excluded.

Moreover, because mAb 2B7 in western blots can bind to TrkC only under nonreducing conditions, we suspect that mAb 2B7 recognizes a TrkC conformation stabilized or influenced by a disulfide bond. This suggests a conformationally sensitive docking site because the epitope contains no Cysteines, and there are no reported disulfide bonds in this region of TrkC.

Biological studies uncovered a unique signal transduction mechanism. mAb 2B7 and its monovalent Fabs mimic NT-3 binding and function. Functional mimicry by mAb 2B7 is indicated by phosphorylation and activation of TrkC, and its downstream signaling partners, and promotion of trophic cell survival. By these criteria, mAb 2B7 is TrkC partial agonist.

The partial agonistic signals induced by mAb 2B7 include MAPK activation (~twofold over baseline) and AKT activation (~fivefold over baseline), and both of these levels of activation are comparable to that resulting form treatment with 100 p*M* NT-3.

However, there are important biological differences between 2B7 and NT-3. First, mAb 2B7 only affords trophic survival but does not induce neuritogenic differentiation in cell lines or in primary neuronal cultures expressing TrkC, and therefore it can be defined as a biased partial agonist. Second, mAb 2B7 does not bind to p75 and is therefore a more selective ligand than NT-3. Third, 2B7 is less potent than NT-3.

Monomeric Ligands Activate TrkC

Tyrosine kinase receptors signal through oligomerization (Kaplan and Stephens, 1994), thus a bivalent ligand was thought to be required for activation. However recently this notion has been challenged and examples exist where genuine monovalent ligands act as agonists of receptor tyrosine kinases, including TrkA (LeSauteur et al., 1996). These data suggest that direct ligand-induced receptor dimerization may not be absolutely required, at least for survival signals.

This seems paradoxical because neurotrophins are homodimeric ligands (Butte et al., 1998) and receptor activation requires dimerization, and/or stabilization of preformed Trk-dimers (Mischel et al., 2002), and/or induction of conformational changes leading to activation (Patapoutian and Reichardt, 2001; Bradshaw and Waksman, 2002).

We speculate that 2B7 Fabs could cause conformational changes in TrkC that induce or stabilize receptor–receptor interactions. The trivial explanation that Fabs are relatively large molecules potentially capable of aggregation is less likely, and there is a precedent reported for small molecule agonists of TrkC ($\sim$700 da) (Zaccaro et al., 2005).

Putative p75•TrkC Interactions

Expression of p75 leads to a reduction of mAb 2B7 binding sites, without a concomitant reduction in TrkC expression. These data indicate that the mAb 2B7 hot spot is either involved in or close to sites of interactions for TrkC•p75. There are at least two mechanisms by which the p75 receptor can affect the availability of the 2B7 epitope in TrkC. It is possible that direct physical association of p75 and TrkC can mask the 2B7 epitope. Physical Trk-p75 interactions were reported in cells overexpressing these receptors (Bibel et al., 1999), but other data have suggested lack of interactions (Wehrman et al., 2007). In addition, allosteric conformational changes may be induced upon TrkC by p75. Indeed, there is a precedent where it was shown that p75 can unmask a cryptic "hot spot" on Trk receptors (Zaccaro et al., 2001), and allosterism has been suggested for FRS-2 association with Trk or FGF receptors (Yan et al., 2002).

We further demonstrate that for blocking mAb 2B7 binding to TrkC, the p75-TM-ICD are not sufficient and that p75-ECD is required. Because the p75-ECD is the domain where ligands can bind p75, we predicted that p75 ligands might alter the "p75-mediated block" of mAb 2B7•TrkC interactions. However, the "p75-mediated block" of mAb 2B7•TrkC interactions appears to be independent of p75 ligands (e.g. they are not required) and furthermore the block is insensitive to the presence of p75 ligands (in this report we used NGF, BDNF, or pro-NT-3, which did not alter the block).

Regulation of TrkC Function by p75

Using mAb 2B7 as a selective TrkC agonist, we show that expression of p75 causes a reduction of 2B7•TrkC signals both in terms of potency (e.g. potency requires higher ligand concentrations) and in terms of efficacy (e.g. the overall strength of the response is lower). This is a striking contrast to NT-3 because expression of p75 can enhance the potency NT-3•TrkC signals without affecting the overall efficacy.

Lower mAb 2B7 potency obviously stems from the fact that p75 causes a reduction in mAb 2B7 binding sites; meaning that fewer TrkC receptors are activated. However, lower efficacy of mAb 2B7 can only be the consequence of the suppression of TrkC signals by p75.

This interpretation would be consistent with a reported reciprocal interplay between TrkA and p75 receptors that regulates signal cascades (Maliartchouk and Saragovi, 1997; Hapner et al., 1998; Maliartchouk et al., 2000; Ivanisevic et al., 2003) and ligand binding (Mahadeo et al., 1994). Notably, however, there is an important difference between TrkA-p75 and TrkC-p75. Suppression of TrkA signals by p75 is responsive to p75 ligands. For example, p75 antagonists enhance TrkA signals (Maliartchouk and Saragovi, 1997; Maliartchouk et al., 2000; Ivanisevic et al., 2003) whereas p75 agonists further suppress TrkA signals (MacPhee and Barker, 1997). In contrast, suppression of TrkC signals by p75 is not responsive to p75 ligands (Ivanisevic et al., 2003).

Uncoupling Survival and Differentiation Signals

The fact that mAb 2B7 can afford trophic survival but completely lacks neuritogenic differentiation

shows that it is possible to uncouple these signals at the level of the ligand acting through a wild type receptor. Previously, this uncoupling has been achieved by mutagenesis of the ICD of receptors. For example, a deletion of five amino acids in the intracellular juxtamembrane region of TrkA abolishes differentiation without affecting survival (Meakin and MacDonald, 1998).

However, it is quite unusual for an agonistic ligand binding at the ectodomain to uncouple signals that are mediated by intracellular adaptor proteins. In this case, we detected efficient pAKT but poor pMAPK signals. This would require a limited conformational change in the receptor activation state such that only some (but not all) adaptor proteins can be activated.

Two scenarios would explain the biology of mAb 2B7 as a biased agonist. First, the ankyrin-rich membrane spanning)/Kidins220 protein (ARMS) protein interacts with Trks through their TM domains, leading to prolonged MAPK signaling and differentiation (Arevalo et al., 2004). TrkC receptors within a putative TrkC-ARMS-p75 complex may not be recognized by mAb 2B7, causing the consequent poor MAPK activation. Second, distinct kinetics of ligand-induced receptor internalization can affect the functional outcome toward neuritogenic differentiation or trophic survival (Saragovi et al., 1998; Segal, 2003), and mAb 2B7 appears to have slower activation kinetics than NT-3.

mAb 2B7 fulfills the criteria of a receptor ligand: selective binding, high affinity, and saturability. Functional assays demonstrate biased agonistic activity. Biased agonists are of great biological interest, but very few have been documented for receptor tyrosine kinases. In particular, it is intriguing that mAb 2B7 is a biased agonistic ligand because it binds to a "hot spot" partially overlapping with NT-3. These data suggest that engaging a TrkC receptor "hot spot" in the juxtamembrane-linker domain can induce survival signals only. Moreover, this "hot spot" can potentially be regulated by expression of p75 either through direct steric hindrance or through direct or indirect changes to the TrkC conformation.

Aberrant expression of *trk*C mRNA has been correlated with neurodegenerative diseases such as Alzheimer's disease (Savaskan et al., 2000), motor neuron diseases, such as amyotropic lateral sclerosis (Saragovi and Gehring, 2000), and some types of cancers (Nakagawara, 2001; Ricci et al., 2001), as well as with photoreceptor disorders (Nag and Wadhwa, 1999) and glaucoma (Rudzinski et al., 2004). Thus, mAb 2B7 or its derivatives may be particularly interesting to study disease mechanisms. Also, mAb 2B7 may be useful in disorders when TrkC-mediated trophic support is desired without inducing neuritic growth, differentiation, or new connections. For validating the use of mAb 2B7 in disease states, it would be of interest to study its survival-promoting effect in motor neurons and other primary cultures, as well as *in vivo*; and this is the focus of our current work.

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