Truncated TrkB receptor-induced outgrowth of dendritic filopodia involves the p75 neurotrophin receptor

Matthias Hartmann[1,2], Tanja Brigadski[1], Kai S. Erdmann[2], Bettina Holtmann[3], Michael Sendtner[3], Frank Narz[2] and Volkmar Leßmann[1,*]

[1]Institute of Physiology and Pathophysiology, Johannes Gutenberg-University Mainz, Duesbergweg 6, 55128 Mainz, Germany
[2]Department of Molecular Neurobiochemistry, Ruhr-University Bochum, Universitätsstraße 150, 44801 Bochum, Germany
[3]Institute for Clinical Neurobiology, Julius-Maximilians University Würzburg, Sanderring 2 - 97070 Würzburg, Germany
*Author for correspondence (e-mail: lessmann@uni-mainz.de)

Accepted 24 August 2004
Journal of Cell Science 117, 5803-5814 Published by The Company of Biologists 2004
doi:10.1242/jcs.01511

Summary

The Trk family of receptor tyrosine kinases and the p75 receptor ($p75^{NTR}$) mediate the effects of neurotrophins on neuronal survival, differentiation and synaptic plasticity. The neurotrophin BDNF and its cognate receptor tyrosine kinase, TrkB.FL, are highly expressed in neurons of the central nervous system. At later stages in postnatal development the truncated TrkB splice variants (TrkB.T1, TrkB.T2) become abundant. However, the signalling and function of these truncated receptors remained largely elusive.

We show that overexpression of TrkB.T1 in hippocampal neurons induces the formation of dendritic filopodia, which are known precursors of synaptic spines. The induction of filopodia by TrkB.T1 occurs independently of neurotrophin binding and of kinase activity of endogenous TrkB.FL. Coexpression of a $p75^{NTR}$ lacking an intracellular domain inhibits the TrkB.T1-induced effect in a dominant negative manner. Steric hindrance of extracellular $p75^{NTR}$ interactions with a specific antibody, or absence of $p75^{NTR}$ with an intact extracellular domain also inhibit this TrkB.T1-induced effect.

We thus propose a novel signalling pathway initiated by neurotrophin-independent extracellular or intramembrane interaction of TrkB.T1 with the $p75^{NTR}$ receptor, which modulates dendritic growth via $p75^{NTR}$ signalling cascades.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/117/24/5803/DC1

Key words: Neurotrophins, Trk, p75 receptor, Filopodia, BDNF, Spines

Introduction

The protein family of mammalian neurotrophins – consisting of NGF, BDNF, NT-3 and NT-4/5 – is known to support survival, differentiation and several forms of synaptic plasticity in CNS neurons (Poo, 2001; Lu, 2003).

The secreted neurotrophins mediate their effects via activation of two different classes of plasma membrane receptors (Patapoutian and Reichardt, 2001). First, each neurotrophin can bind as a homodimer to its specific Trk tyrosine kinase receptor (NGF binds to TrkA, BDNF and NT-4/5 to TrkB and NT-3 preferentially binds to TrkC). Secondly, all neurotrophins are known to bind with equal specificity to the p75 neurotrophin receptor ($p75^{NTR}$). Upon binding of neurotrophins, Trks activate a tyrosine kinase signalling cascade that accounts for numerous neurotrophin-dependent biological effects in neurons (Patapoutian and Reichardt, 2001). The $p75^{NTR}$ modulates Trk signalling and can even initiate cellular responses independently from Trk signalling (see Kaplan and Miller, 2000). This is accomplished via protein-protein interactions with a set of intracellular binding partners including e.g., RhoA (a small G-protein involved in neurite outgrowth), NRAGE, NRIF and TRAFs (Roux and Barker, 2002). In addition, $p75^{NTR}$ can initiate the production of ceramide via intermediate activation of neutral sphingomyelinase (Dobrowsky and Carter, 1998). This pathway has recently been implicated in p75-mediated growth of neuronal processes in hippocampal neurons (Brann et al., 1999; Brann et al., 2002).

The rat TrkB tyrosine kinase receptor for BDNF and NT-4/5 (TrkB.FL) can also exist as alternatively spliced truncated receptor isoforms TrkB.T1 and TrkB.T2, respectively, which lack the intracellular kinase domain (Klein et al., 1990; Middlemas, 1991). Although it is recognised that the TrkB.T1 receptor is the dominant TrkB isoform expressed in the adult rodent CNS (Cabelli, 1996), the physiological function of these truncated receptors are still not well established and intracellular downstream effectors have long remained elusive (see Baxter et al., 1997; Kryl and Barker, 2000; Rose et al., 2003). Most evidence suggests a role of TrkB.T1 and T2 in ligand presentation or in ligand scavenging for TrkB.FL (Bothwell, 1995; Biffo et al., 1995), or as a dominant negative inhibitor of TrkB.FL signalling via formation of heterodimers (Eide et al., 1996; Ninkina et al., 1996).

Since one of the classical actions of neurotrophins is to promote neuronal fibre outgrowth (Levi-Montalcini and

Hamburger, 1953), it was an intriguing recent finding, that overexpression of TrkB.T1 receptors in cortical slice cultures can initiate dendritic branching, which is negatively regulated by phosphorylated TrkB.FL receptors coexpressed in the same cells (Yacoubian and Lo, 2000). Comparable effects were reported for filopodial growth induced by ectopic expression of TrkB.T1 in a neuroblastoma cell line (Haapasalo et al., 1999). However, both studies did not delineate the mechanism of these TrkB.T1-induced effects.

In order to investigate the underlying signalling mechanism of TrkB.T1-induced process outgrowth in postnatal rat hippocampal neurons, we overexpressed GFP-tagged versions of the different TrkB receptor isoforms in neuron microcultures. We found that overexpression of TrkB.T1 and TrkB.T2 induced a two- to threefold increase in the number of dendritic filopodia. This effect was independent of neurotrophin binding and was also seen with a mutant TrkB.T1 receptor that completely lacked the cytoplasmic tail. Coexpression of TrkB.FL had a dominant negative effect on TrkB.T1-induced filopodial growth. As revealed by application of an antibody that binds to the extracellular epitope of the $p75^{NTR}$ and that inhibits filopodia formation, this TrkB.T1-induced growth of filopodia involves $p75^{NTR}$ signalling. Accordingly, the TrkB.T1-induced effect is inhibited by coexpression of a dominant negative $p75^{NTR}$, which lacks the cytoplasmic domain, and is significantly reduced in neurons derived from exon III $p75^{NTR}$ knockout mice.

These data add a new facet to the functions of NT receptor signalling, suggesting an activation of certain aspects of $p75^{NTR}$ signalling by interaction with non-liganded truncated TrkB receptors. This interaction could play a role in promoting synapse formation via dendritic filopodia.

Materials and Methods

Construction of TrkB expression vectors

To facilitate detection of protein expression in living cells, green fluorescent protein (GFP) was fused C-terminally, i.e. to the intracellular domain, of the three known rat TrkB isoforms TrkB.FL, TrkB.T1 and TrkB.T2 (Middlemas et al., 1991). TrkB cDNAs used as templates for PCR amplification were kindly provided by D. Middlemas. Oligonucleotides were synthesised for the respective 3′ and 5′ termini to allow restriction digestion after the PCR reaction and deletion of stop codons in case of GFP fusion proteins (e.g. 3′ primers: TrkB.FL-GFP, GATGGATCCCGGCCTAGGATGTCCAGGTAG; TrkB.T1-GFP, GATGGATCCCGCCCATCCAGGGGGATCTTATG; TrkB.T2-GFP, GATGGATCCCGAGAAGCAAAATAAGCACACTTCTG; TrkB.T1ΔICD-GFP, GATGGATCCGTCGCCAACTTGAGCAGA-AGC. 5′ primer for all four constructs: ACGAATTCGCCACCAT-GTCGCCCTGGCCGAGGTG). Amplified fragments were digested with *Eco*RI and *Bam*HI and ligated into the pEGFP-N1 or pDsRed2-N1 vector (CLONTECH Inc.). Unfused controls (TrkB.T1, wt TrkB.FL) were constructed accordingly without removal of the stop codon. In order to examine the role of $p75^{NTR}$ signalling, a $p75^{NTR}$-GFP fusion protein and the deletion construct p75ΔICD (fused to DsRed) were cloned using a similar strategy. All constructs were verified by sequencing. Expression of all constructs used in this study was driven by CMV promoters.

Cell culture

COS7 and PC12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing either 10% fetal calf serum (COS7) or 10% horse serum and 5% fetal calf serum (PC12) and supplemented with penicillin and streptomycin (37°C in 10% CO_2). Upon reaching confluence, cells were passaged 1:3 (PC12) or 1:7 (COS7). For transfection and fluorescence imaging, cells were plated on uncoated glass coverslips with a density of 100,000 cells/cm^2. Transfection was performed 1 day after plating, fluorescence imaging 1-2 days after transfection.

Hippocampal microcultures

Dissociated postnatal rat hippocampal microcultures were prepared as described previously (Lessmann and Heumann, 1998), with minor modifications. Primary postnatal (P0-P2) neocortical astrocytes were isolated and cultured on glass cover slips at low density in DMEM medium containing 10% FCS, to yield astrocyte islands of 100-300 μm in diameter after 7-14 days in vitro (DIV). Postnatal rat hippocampal neurons were plated at a density of 5-20 neurons per astrocyte island onto the astrocyte coverslips, and neurons were cultured in serum-free medium (Neurobasal with 2% B27; Gibco). After 4 days, 4 μM ARAC was added to inhibit glial proliferation. Cultures from $p75^{NTR}$ knockout mice were prepared accordingly from the offspring of homozygous male and female $p75^{NTR}$ knockout mice (Lee et al., 1992), obtained from a local breeding colony.

Transfection

Rat hippocampal microcultures were transfected with the expression plasmids at 8-9 DIV, using the calcium phosphate precipitation method as described previously (Haubensak et al., 1998). After transfection, 4% fetal calf serum (FCS) was added. Cells were used for experiments 2-3 days after transfection (10-11 DIV). Cultures from $p75^{NTR}$ knockout mice and from C57/BL6 wild-type mice were transfected at 7-8 DIV with TrkB.T1-GFP and GFP, respectively (1 μg), and cotransfected with DsRed2 plasmid (2 μg). No FCS was added. Quantification of filopodia in these experiments was performed by a person blind to the identity of the cultures.

PC12 and COS7 cells were transfected with the polyethylenimine (PEI) method as described by others (Lambert et al., 1996). Briefly, 5 μg DNA per 35 mm dish were diluted in 125 μl of 0.15 M NaCl, mixed with 15 μl PEI solution (900 μg/ml 50% PEI 800 kDa, pH 6.5) and 110 μl 0.15 M NaCl, incubated for 10 minutes at room temperature and added to 1 ml serum-free DMEM. After incubating the cells with this solution for 3 hours (37°C, 10% CO_2), the medium was changed to normal cell culture medium (see above). For the fibre outgrowth assay, 20-30 green fluorescent cells were evaluated per coverslip.

Fluorescence imaging

Coverslips with transfected cells were transferred into Petriperm dishes (Vivascience) with folio bottoms and observation of fluorescence signals was performed as described previously (Hartmann et al., 2001).

Cells were inspected through high aperture oil immersion objectives (40×, NA: 1.0; 100×, NA: 1.35) of an inverted epifluorescence microscope (Olympus IX 70). GFP fluorescence was detected with narrow excitation (450-490 nm) and emission (dichroic mirror: 495 nm; 500-550 nm) band pass filters. Red fluorescence was detected with a custom built filter set (excitation: 530-550 nm; dichroic mirror: 570 nm; emission: 590-650 nm). Illumination of the probe was restricted to image capture and controlled with an electronic shutter device (Uni-Blitz). Pictures were captured with a digital CCD camera (Sensys 1401E, Photometrics). Data acquisition and analysis was performed with MetaView software (Universal Imaging). Brightness and contrast of images were adjusted to make use of the dynamic range of colour representation. All images of a given figure were treated equally.

Immunocytochemistry

Coverslips of transfected cells were fixed (4% formaldehyde in PBS) and permeabilized (0.1% Triton X-100) using standard procedures (see Haubensak et al., 1998). Tau antibody (mouse, 1:200; Boehringer Mannheim), TrkB.T1 antibody (TrkB[TK-], rabbit, 1:200; Santa Cruz Biotechnology) and MAP2 antibody (mouse, 1:200; Sigma) were incubated for 1 hour at room temperature (RT) followed by incubation (RT, 1 hour) with Alexa Green-coupled anti-rabbit secondary antibody or Alexa Red-coupled anti-mouse secondary antibody (both 1:1000; Molecular Probes). Where required, polymerized filamentous actin was stained by incubating with Phalloidin-TRITC (1:100, Sigma) for 20 minutes (at RT).

Sholl analysis

To measure the complexity of the dendritic tree of cultured neurons, Sholl analysis was performed on single transfected neurons on microislands 2-3 days after transfection (10-11 DIV). Imaginary circles with radii in increments of 5 μm up to 30 μm were drawn around the cell body, and the number of intersections with fluorescent neurites of the same neuron were counted.

Quantification of the density of filopodia

To determine the effect of TrkB.T1 expression on filopodia formation, the filopodic density of single transfected neurons on microislands was determined 2 days after transfection (10 DIV). Of every neuron under examination a 40 μm section of a typical dendrite that was in the plane of focus was chosen for counting. For these sections the number of protrusions between 1 and 10 μm in length were determined. Inhibitors, ligands etc. were applied on the day of transfection at concentrations as indicated. All results are given as mean values ± s.e.m. Statistical significance was determined using the two-tailed Student's *t*-test. The absolute numbers of filopodia were dependent on experimental conditions. The batch of FCS used for plating, the person responsible for counting and the necessity to add FCS after transfection to rat but not to mouse neurons were the most sensitive factors for the count of filopodia. Therefore negative (GFP transfected or TrkB.FL transfected) and positive controls (TrkB.T1-GFP) were included in all experiments. Only data from experiments with identical conditions were pooled.

Reagents

K252a and neurotrophins were obtained from Alomone Labs, TrkB-Fc receptor bodies from R&D Systems and MC192 monoclonal antibody was purchased from Chemicon. Intact biological activity of neurotrophins, of TrkB-Fc receptor bodies, and of k252a was tested at regular intervals in a PC12 cell fibre outgrowth assay (see supplementary material).

Intact biological function of neurotrophins

The intact biological function of the exogenously added neurotrophins in our experiments (i.e. NGF, BDNF, NT-4/5, 100 ng/ml each), of the Trk kinase inhibitor k252a (200 nM) and of the TrkB receptor bodies (TrkB-Fc; 1 μg/ml) was tested at regular intervals using the above mentioned PC12 cell assay (see Fig. S1 in supplementary material). GFP-transfected PC12 cells (endogenously expressing TrkA) were used to test NGF and k252a. TrkB.FL-GFP-expressing PC12 cells were employed to test BDNF and NT-4/5. TrkB.FL-expressing PC12 cells cotransfected with GFP were used to examine the intact function of TrkB-Fc. Only neurotrophin batches yielding maximal stimulation (i.e. >50%, compare Fig. S1 in supplementary material) of fibre outgrowth at a concentration of 100 ng/ml were used for experiments with neurons. The Trk kinase inhibitor k252a (200 nM) significantly inhibited NGF-induced fibre outgrowth and proliferation of NGF-treated (100 ng/ml) PC12 cells (data not shown).

Results

In order to visualize the localization and assess the biological function of truncated TrkB receptors in hippocampal neurons we constructed GFP-tagged versions of rat TrkB.FL, TrkB.T1 and TrkB.T2 receptors. The GFP tag was fused via a seven amino acid flexible spacer to the C terminus of the different TrkB receptor isoforms (Fig. 1). These GFP-labeled constructs allowed us to directly identify transfected cells and to ascertain intact cell membrane targeting of the overexpressed constructs in each cell investigated (Klau et al., 2001). The intact biological function of the GFP-tagged TrkB.FL receptor (TrkB.FL-GFP) was investigated by testing ligand-induced process formation in TrkB.FL-GFP-transfected PC12 cells. This cell line is known to show fibre outgrowth when ectopically expressed TrkB receptors are activated by BDNF (Ip et al., 1993b; Fanger et al., 1995). One day after transient transfection with our TrkB-GFP construct, BDNF or NT-4/5 (100 ng/ml; 3 days) induced fibre outgrowth in these PC12 cells to an extent, which was indistinguishable from, (i) the respective process formation obtained following overexpression of untagged TrkB.FL, and, (ii) from NGF-induced fibre outgrowth in the same cells (Fig. S1 in supplementary material). This is in line with the intact ligand-induced tyrosine autophosphorylation of TrkB-GFP observed in COS7 cells (data not shown) (see also Watson et al., 1999).

Upon transfection (at 8 DIV) of postnatal rat hippocampal neurons with the TrkB.T1-GFP and TrkB.FL-GFP constructs, we observed a correct plasma membrane localization of both receptor isoforms in the soma and in the processes of living

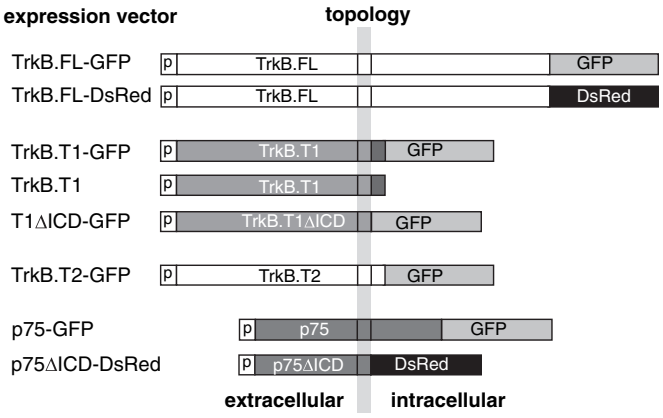

Fig. 1. Topology of cloned TrkB and p75 fusion proteins. Green fluorescent protein (GFP) and red fluorescent DsRed was fused C-terminally to full length rat TrkB (TrkB.FL), yielding TrkB.FL-GFP and TrkB.FL-DsRed. Similarly, the truncated TrkB isoforms were constructed to yield TrkB.T1-GFP and TrkB.T2-GFP. As a control, unfused TrkB.T1 was cloned into the same vector. The intracellular domain of TrkB.T1 (black bar) was deleted in the construct T1ΔICD-GFP. C-terminal fusion of GFP to rat p75^{NTR} yielded p75-GFP. A dominant negative p75^{NTR} (lowermost construct) was generated by fusing DsRed to the transmembrane domain of p75^{NTR}. The grey vertical bar shows the location of the plasma membrane; the extracellular space is to the left. The p indicates the pre sequence directing the receptor mRNAs to the rough ER.

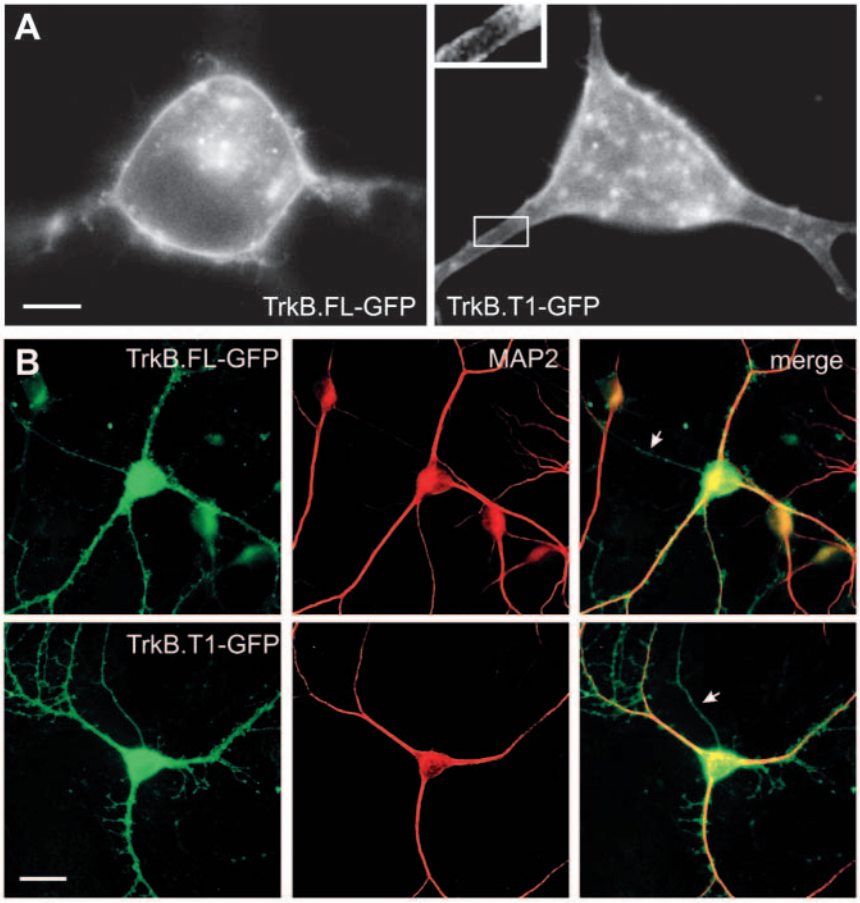

Fig. 2. TrkB.FL-GFP and TrkB.T1-GFP are localized in the plasma membrane of hippocampal neurons and are targeted to dendrites and axons. (A) Hippocampal neurons, transfected with TrkB.FL-GFP (left) and TrkB.T1-GFP (right) expression plasmids at 8 days in vitro (DIV), showed a clear accumulation of GFP fluorescence at the plasma membrane (2 days after transfection). (B) Immunocytochemistry of transfected neurons with an antibody against the dendritic marker MAP2 (at 16 DIV, 8 days after transfection). TrkB.FL-GFP and TrkB.T1-GFP in hippocampal neurons (green), were targeted to both, dendrites (MAP2-positive, red) and axons (MAP2-negative, arrows). Filopodia (small protrusions) were restricted to dendrites, whereas the appearance of axons was generally smooth. Scale bars: 5 μm (A); 20 μm (B).

neurons (Fig. 2A). As expected, a substantial fraction of GFP-labeled TrkB.FL and of TrkB.T1 receptors was also found in intracellular compartments (Du et al., 2000; Haapasalo et al., 2002), the relative abundance of which was not further analyzed here. As indicated by colocalization with the dendritic marker MAP2 (Fig. 2B), both receptors were targeted to dendritic compartments (see also Kryl et al., 1999). In addition, axonal targeting was also evident in all neurons investigated, as indicated by the GFP fluorescence in MAP2-negative thin processes of transfected neurons (see arrows in Fig. 2B), and from colocalization with the axonal marker protein Tau (see Fig. S3 in supplementary material). Similar results were obtained when TrkB.T2 receptors were overexpressed (data not shown). These results suggest general neuronal (non-polarized) expression of the different TrkB receptor variants in our hippocampal neurons.

Interestingly, the expression of TrkB.T1-GFP in postnatal rat hippocampal neurons induced a highly significant ($P<10^{-5}$) roughly twofold increase in the number of dendritic filopodia, as compared to TrkB.FL-GFP and wt-GFP-overexpressing controls (see Fig. 3). For these and all further experiments only cells with a clear plasma membrane localization of the overexpressed receptors were selected, however, owing to image processing procedures necessary to visualize thin processes, membrane localization of the receptors is not evident in these pictures. Staining with TRITC-labeled phalloidin revealed the presence of filamentous actin in these membrane protrusions, which is a characteristic marker for dendritic filopodia. These filopodia typically give rise to the formation of synaptic spines at later stages of development (see Harris, 1999). As revealed by Sholl analysis, the number of primary dendrites and of proximal dendritic branches was not significantly affected in these experiments (see Fig. 3C), corroborating the selective effect on filopodia. In spite of the unpolarized expression of TrkB.T1 in our neurons, we did not observe a similar effect of TrkB.T1 on the growth of axonal filopodia.

Given the membrane targeting of both receptors (see Fig. 2), the selective induction of filopodia in TrkB.T1-GFP-overexpressing versus TrkB.FL-GFP-overexpressing cells clearly indicates true induction of processes rather than just highlighting of processes by membrane targeted GFP.

This interpretation is supported by control experiments in which filopodia formation was analyzed by the red fluorescence of coexpressed DsRed protein in neurons transfected with either TrkB.T1-GFP or wt-GFP: mouse hippocampal neurons were cotransfected at 8 DIV with either TrkB.T1-GFP and DsRed (3:1) or with GFP and DsRed (3:1). Two days posttransfection, dendritic filopodia were counted in the red fluorescent channel (DsRed) for both conditions. There was a roughly twofold visible increase in TrkB.T1-GFP-expressing neurons (significantly different with $P<0.0001$; see Fig. S2 in supplementary material). These results indicate that the TrkB.T1-induced induction of filopodia is also visible when cytosolic DsRed fluorescence is evaluated in both conditions. This is also evident from the largely overlapping fluorescence of TrkB-T1-GFP and DsRed fluorescence in the same cells. Thus, either GFP- or TrkB.FL-GFP-expressing cells could be used as negative control in all subsequent experiments.

Further control experiments employing overexpression of untagged TrkB.T1 receptors and analysis of filopodia by the red fluorescence of coexpressed DsRed demonstrated a similar induction of filopodia also for untagged TrkB.T1 receptors: rat hippocampal neurons were cotransfected at 8 DIV with TrkB.T1 receptors (either TrkB.T1-GFP or wt TrkB.T1) and a DsRed expression plasmid (DNA ratio TrkB.T1:DsRed, 3:1). As a negative control, sister cultures were cotransfected with GFP and DsRed (DNA ratio, 3:1). Two days after transfection, dendritic filopodia were counted in the three groups in the red

fluorescent channel (DsRed). GFP-tagged and wt TrkB.T1 receptor both showed a 1.6-fold increased number of dendritic filopodia compared to the negative control (see Fig. S4 in supplementary material; both TrkB.T1 groups significantly different from GFP with $P<0.001$). These results indicate that induction of filopodia by TrkB.T1 is not an artefact of the GFP tag. This rules out that the GFP tag of TrkB.T1 was responsible for the indcution of filopodia.

A similar increase in the growth of dendritic filopodia was observed when TrkB.T2-GFP was overexpressed in hippocampal neurons (data not shown). However, since TrkB.T1 is more highly conserved between species and is the only truncated receptor in humans (Biffo et al., 1995; Shelton et al., 1995), all subsequent experiments were performed with the TrkB.T1-GFP construct.

To assess the signaling mechanism of the TrkB.T1-induced outgrowth of dendritic filopodia, we examined the effects of endogenously released and of exogenously added TrkB receptor ligands, respectively (Fig. 4). In order to scavenge endogenously released BDNF and/or NT-4/5 in our cultures, 0.4 µg/ml TrkB-Fc receptor bodies (Shelton et al., 1995) were added at the day of transfection (8 DIV). This treatment had no effect on the magnitude of filopodial growth in either TrkB.T1-GFP- or TrkB.FL-GFP-overexpressing neurons. Likewise, the TrkB.T1-GFP-induced growth of filopodia and the basal levels of filopodia formation in TrkB.FL-GFP-expressing and wt-GFP-expressing neurons was not affected by the exogenous application of BDNF or NT-4/5 (100 ng/ml). These results indicate that the TrkB.T1-induced growth of filopodia occurs independently from neurotrophin binding. In addition, these data demonstrate that the basal formation of dendritic filopodia in our cultures is independent of ligand-induced signalling via endogenously expressed or overexpressed full length TrkB receptors.

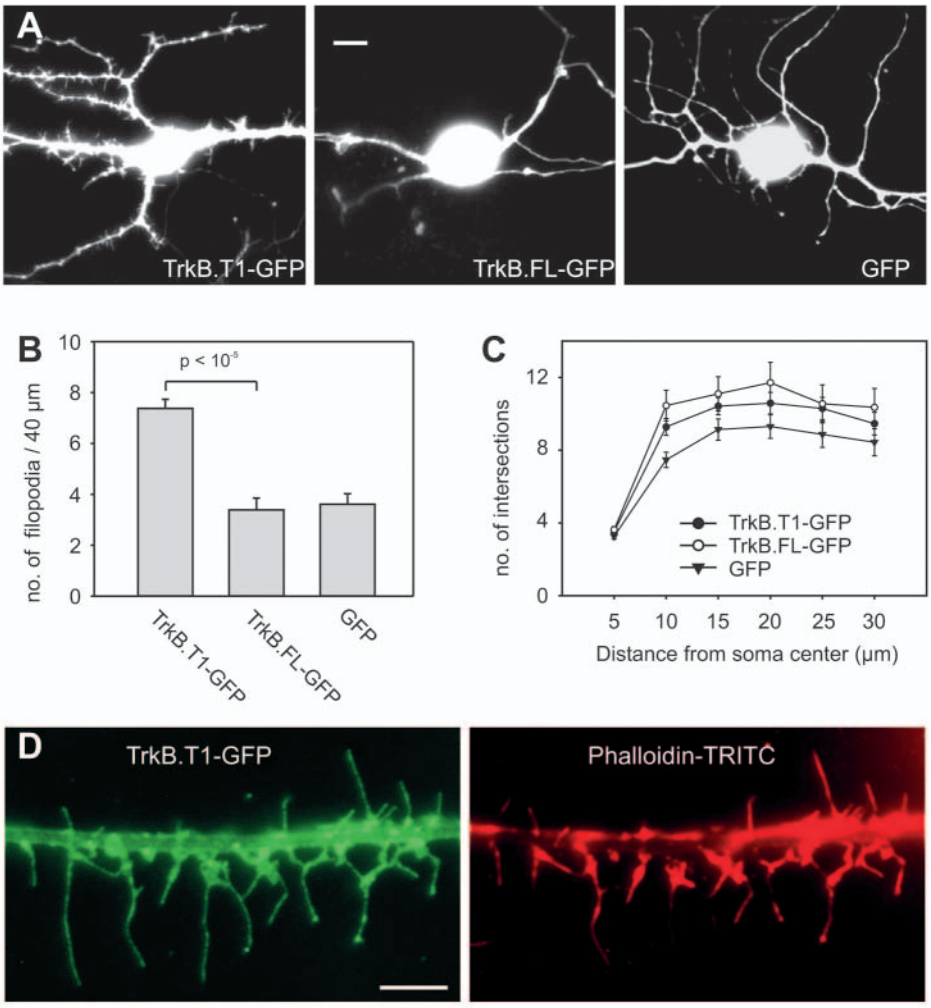

Fig. 3. TrkB.T1 expression in hippocampal neurons increases the number of dendritic filopodia. (A) Typical examples of the morphology of TrkB.T1-GFP-, TrkB.FL-GFP- and GFP-expressing hippocampal neurons (at 11 DIV, 3 days after transfection). The increased number of filopodia in TrkB.T1-GFP expressing neurons lead to a ruffled appearance (left) compared to the much smoother TrkB.FL-GFP and GFP controls. (B) The increase in density of filopodia was highly significant (n=26-91 cells, 5-13 independent experiments, $P<10^{-5}$). (C) Sholl analysis of all neurons from B. The expression of TrkB.T1-GFP, had no significant effect on the complexity of the dendritic tree. (D) Typical dendrite of a TrkB.T1-GFP expressing hippocampal neuron (green, at 10 DIV). Phalloidin-TRITC staining (red) of TrkB.T1-GFP-induced filopodia revealed their filamentous actin content, which is characteristic for filopodia. Scale bars: 10 µm (A); 5 µm (D).

The independence of the TrkB.T1-induced formation of dendritic filopodia from full length TrkB signalling was further corroborated by experiments wherein the signalling of Trk receptors was completely inhibited by application of supramaximal concentrations of the Trk kinase inhibitor K252a (200 nM, Fig. 4B): again, this treatment did not affect either the TrkB.T1-induced formation of filopodia or the basal levels of filopodia in control cultures expressing endogenous levels of TrkB.FL. Thus, the TrkB.T1-induced formation of filopodia cannot be explained by an interference of the overexpressed TrkB.T1 with the full-length TrkB tyrosine kinase signalling. Rather, our results suggest a novel independent signalling of non-liganded TrkB.T1 receptors in hippocampal neurons.

To examine whether the short intracellular tail of the TrkB.T1 receptor, which is highly conserved among species, is necessary to observe the effect on filopodial growth, we generated a mutant lacking all of the intracellular domain of TrkB.T1 (TrkB.T1ΔICD-GFP; see Fig. 1). Again, the GFP tag allowed us to directly ascertain that this mutant receptor was targeted to the membrane (data not shown). To our surprise, overexpression of this construct yielded induction of dendritic filopodia in hippocampal neurons to an extent that was indistinguishable from the TrkB.T1-GFP-induced effect (not significantly different with $P>0.14$; Fig. 4C). The most likely explanation of

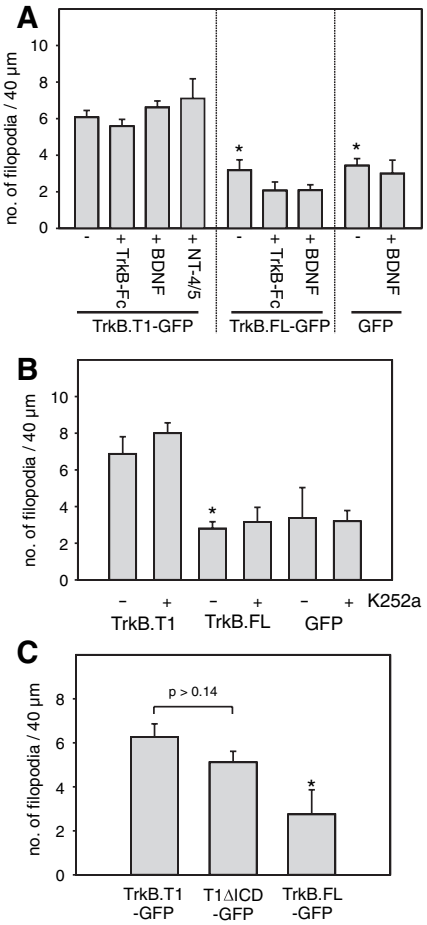

Fig. 4. The induction of filopodia by TrkB.T1 is independent from ligand binding, TrkB kinase activity, and from the intracellular domain of TrkB.T1. (A) Hippocampal neurons were transfected with TrkB.T1-GFP, TrkB.FL-GFP or GFP expression plasmids. On the day of transfection, either TrkB ligand (BDNF or NT-4/5) was added (100 ng/ml), or endogenously released ligand was scavenged with TrkB-Fc receptor bodies (0.4 µg/ml). The density of filopodia was determined 2 days later. Neither exogenous application of ligand nor scavenging of endogenously released ligands with TrkB-Fc receptor bodies influenced the density of dendritic filopodia (n=10-50 cells; two to five independent experiments, *$P<10^{-4}$, compared with TrkB.T1-GFP positive control). (B) K252a (200 nM), did not affect the growth of filopodia, indicating the lack of an effect of Trk kinase signalling. *Significantly different from TrkB.T1-GFP with $P<0.05$. (C) The induction of filopodia by a deletion mutant of TrkB.T1 without intracellular domain (T1ΔICD-GFP) was not significantly different ($P>0.14$) from the TrkB.T1-GFP-induced effect. The increase in filopodia caused by T1ΔICD-GFP is statistically significant compared to TrkB.FL-GFP transfected controls (*$P<0.05$, n=8-26 cells).

this result is, that an extracellular or intramembrane interaction of the TrkB.T1 receptor with an unidentified membrane protein is sufficient for the induction of filopodia.

Since the truncated TrkB receptors are known to form heterodimers with TrkB.FL (Eide et al., 1996; Ninkina et al., 1996) we reasoned that unliganded TrkB.FL could be the interacting membrane protein for TrkB.T1 in our cells. Thus, we coexpressed TrkB.FL-DsRed together with TrkB.T1-GFP (ratio TrkB.FL:TrkB.T1 DNA: 3:1) and compared the formation of dendritic filopodia in these coexpressers to neurons expressing only TrkB.T1-GFP in the same culture dishes (Fig. 5). In order to definitely rule out endogenous Trk kinase signalling, 200 nM K252a was added at the day of transfection. Instead of disclosing a role for a signalling competent TrkB.FL/TrkB.T1 heterodimer, these experiments revealed a dominant negative effect of coexpressed TrkB.FL on the TrkB.T1-induced formation of filopodia. These results clearly indicate that the full-length TrkB receptor can not be the transducer of TrkB.T1-dependent formation of dendritic

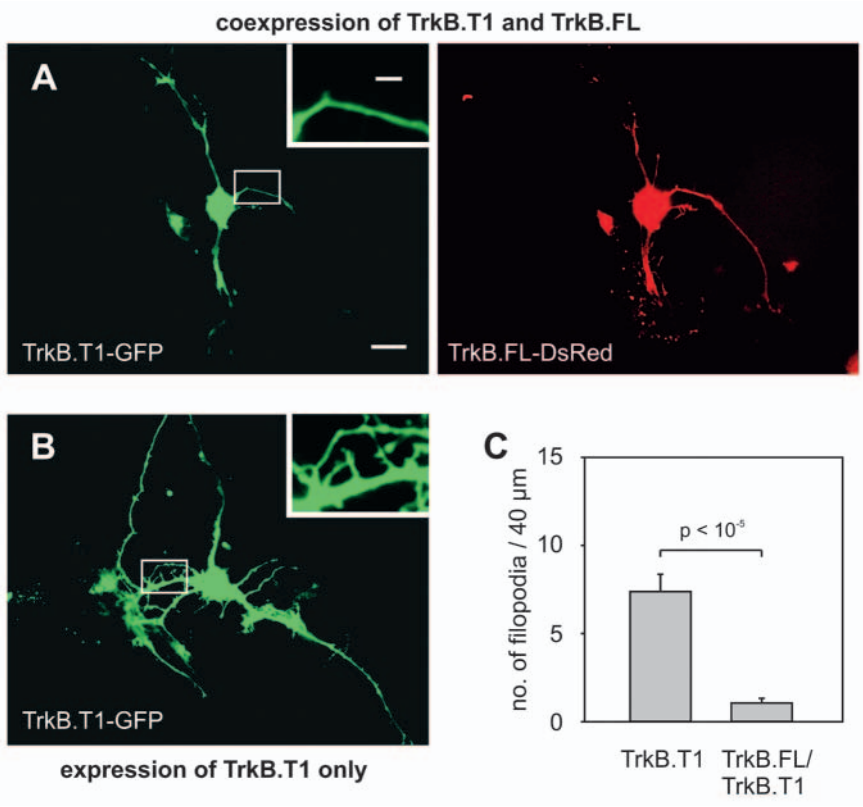

Fig. 5. Expression of TrkB.FL inhibits the TrkB.T1-induced growth of filopodia in a dominant negative fashion. Hippocampal microcultures were cotransfected with TrkB.T1-GFP and TrkB.FL-DsRed (relative amounts of DNA: 1:3). (A) A typical neuron, expressing both TrkB.T1-GFP and TrkB.FL-DsRed. Note the smooth dendritic surface, compared to B, a neuron expressing TrkB.T1-GFP only. (Insets in A an B show boxed areas at higher magnification.) (C) The reduction in the density of filopodia by coexpression of TrkB.FL is highly significant compared to controls expressing TrkB.T1 only ($P<10^{-5}$ n=16-22 cells; three independent experiments). Both groups of neurons from a given experiment were located on the same glass cover slip. All experiments were performed in the presence of 200 nM K252a. Therefore, TrkB receptor tyrosine kinase activity is not required for the dominant negative action of TrkB.FL. Scale bars: 20 µm (A); 5 µm (inset).

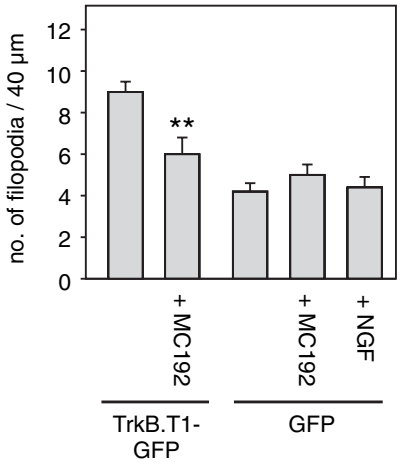

Fig. 6. TrkB.T1-induced formation of filopodia is dependent on p75^{NTR} but can not be mimicked by application of NGF. Application of 8 µg/ml MC192 (a monoclonal antibody that binds to an extracellular epitope of p75^{NTR}) to TrkB.T1-overexpressing neurons reduced the outgrowth of filopodia to control levels (**significantly different from TrkB.T1-GFP; P<0.01). Basal levels of filopodia remained unaffected (see GFP controls). Application of 100 ng/ml NGF to GFP transfected controls does not mimic the effect of TrkB.T1 expression (n>30cells; five experiments).

filopodia. However, the dominant negative effect of the TrkB.FL in these experiments suggested that both receptor isoforms might compete for a common downstream effector.

Since the p75^{NTR} is known to physically interact with TrkB.T1 and TrkB.FL receptors (Bibel et al., 1999), we hypothesized that the TrkB.T1-induced outgrowth of filopodia in our hippocampal cultures could be mediated by interaction with the p75^{NTR}. A contribution of p75^{NTR} signalling is in fact supported by experiments making use of the highly specific monoclonal p75 antibody MC192. This antibody binds to an extracellular epitope of the p75^{NTR}, which is not involved in ligand binding but rather sterically inhibits (at high antibody concentrations of 8 µg/ml) the interaction of p75^{NTR} with TrkA receptors in the outer membrane leaflet (Barker and Shooter, 1994). Thus, in the case of the physical extracellular interaction of TrkB.T1 and p75^{NTR} in our experiments we expected to see an MC192-dependent inhibition of filopodial growth. In fact, incubation of TrkB.T1-GFP-expressing neurons with MC192 (8 µg/ml) significantly reduced (P<0.01) the T1-induced formation of filopodia in our hippocampal cultures to nearly control levels, whereas basal levels of filopodia in wt-GFP controls remained unaffected (Fig. 6). Lower concentrations of MC192 (0.2 µg/ml), which are known to promote synergistic interactions between p75^{NTR} and Trk receptors (Maliartchouk and Saragovi, 1997) failed to show any effect on filopodial growth in our cultures (data not shown). Interestingly, overexpression of p75^{NTR} in hippocampal neurons resulted in the

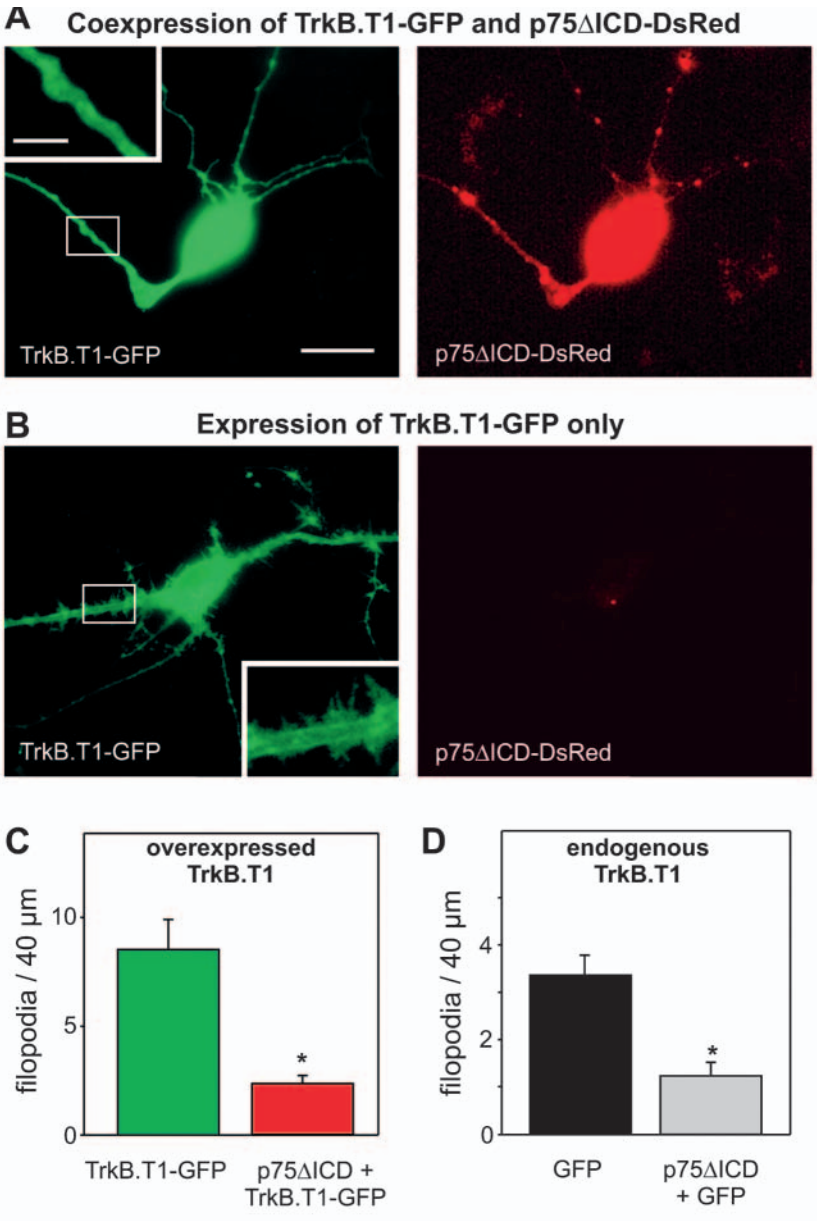

Fig. 7. Coexpression of a dominant negative p75^{NTR} variant (p75ΔICD-DsRed) inhibits the TrkB.T1-induced growth of filopodia. Neurons were cotransfected with TrkB.T1-GFP and p75ΔICD-DsRed (relative amounts of DNA: 3:1). (A) Typical neuron, expressing both, TrkB.T1-GFP and p75ΔICD-DsRed. Note the lack of filopodia compared with the neuron expressing TrkB.T1-GFP only (on the same coverslip) shown in B. Insets in A and B show boxed areas at higher magnification. (C) The reduction in the density of filopodia by coexpression of p75ΔICD-DsRed is highly significant (P<0.0001). (D) Neurons were cotransfected with GFP and p75ΔICD-DsRed (relative amounts of DNA: 3:1). Cells coexpressing p75ΔICD-DsRed showed a significant (P<0.0001) reduction of filopodia compared with neurons expressing GFP only, on the identical coverslips, highlighting the physiological significance of TrkB.T1-induced filopodial growth (n=13-30 cells from three independent experiments). Scale bars: 20 µm (A); 5 µm (inset).

induction of filopodial growth on its own (p75: 5.5±0.5 filopodia per 40 μm dendrite; TrkB.T1: 6.3±0.6; TrkB.FL: 2.8±1.1, significantly different with $P<0.01$). However, in many cases the overexpression of $p75^{NTR}$ also induced neuronal death, which is in line with neurotrophin-independent induction of neuronal death via the intracellular domain of $p75^{NTR}$ in vivo (Majdan et al., 1997).

Since TrkA receptors are absent from hippocampal neurons, application of NGF can be used to selectively activate $p75^{NTR}$ signalling in these cells (Ip et al., 1993a; Brann et al., 1999; Friedman, 2000). To examine whether NGF-induced $p75^{NTR}$ activation is sufficient to induce filopodia, we stimulated GFP-transfected neurons with NGF (100 ng/ml). Interestingly, NGF failed to reproduce the TrkB.T1-induced growth of filopodia (Fig. 6), suggesting that partially different signalling mechanisms are at work downstream of the $p75^{NTR}$, depending on whether NGF or TrkB.T1 is interacting.

Altogether, these data suggested that the TrkB.T1-induced formation of filopodia is transduced via neurotrophin-independent interaction of the extracellular or transmembrane domains of $p75^{NTR}$ and TrkB.T1 receptors, initiating downstream activation of certain aspects of $p75^{NTR}$ signalling.

Intriguingly, coexpression of TrkB.T1-GFP with a mutant $p75^{NTR}$, which lacks the intracellular domain (p75ΔICD-DsRed), indeed inhibits the TrkB.T1-induced effect in a dominant negative manner: the formation of dendritic filopodia in these coexpressers was reduced fourfold, when compared to neurons expressing only TrkB.T1-GFP, in the same culture (Fig. 7). It is of note, that in these experiments TrkB.T1-GFP-expressing cells were collected for both groups from identical coverslips. Thus, the observed differences in filopodial growth are only related to the presence/absence of p75ΔICD-DsRed in the cells, ruling out any bias in our analysis. This experiment provides strong evidence for the model of endogenous $p75^{NTR}$ being a downstream mediator of TrkB.T1-induced filopodia formation. Importantly, p75ΔICD-DsRed also significantly reduced basal levels of filopodia formation in wt-GFP-expressing controls (Fig. 7D), thus suggesting that endogenously expressed TrkB.T1 contributes to basal filopodia formation. In order to test for endogenous expression of TrkB.T1 in our neurons we performed immunocytochemistry with an antibody that reacts selectively with truncated TrkB receptors (anti-TrkB[TK–]). The antibody recognized truncated TrkB receptors in astrocytes (see also Rose et al., 2003) and in neurons of our hippocampal microcultures (Fig. 8). This supports the view that endogenous TrkB.T1 could account for basal levels of filopodial growth in our cultures (Fig. 7D).

In order to further establish TrkB.T1-induced signalling via $p75^{NTR}$ we performed experiments in hippocampal microcultures from exon III $p75^{NTR}$ knockout mice (Lee et al., 1992), which show a strong reduction, albeit not a complete deletion of all $p75^{NTR}$ isoforms (von Schack et al., 2001). Importantly, the TrkB.T1-induced formation of filopodia was significantly reduced in these cultures compared to matched wild-type controls (TrkB.T1 in p75 knockout: 14.5±0.9 filopodia per 40 μm dendrite; TrkB.T1 in wt: 20.0±1.1; significantly different with $P<0.0005$, see Fig. 9). Nevertheless, the TrkB.T1-induced formation of filopodia was not abolished in these $p75^{NTR}$ knockout cultures. This suggests that the remaining extracellularly truncated $p75^{NTR}$ in these mice (von Schack et al., 2001) can partially substitute for the full length $p75^{NTR}$.

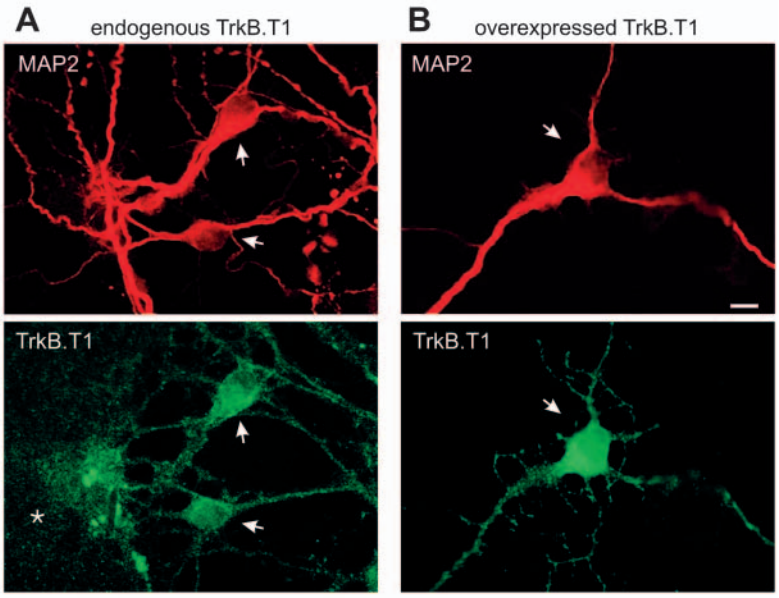

Fig. 8. TrkB.T1 is endogenously expressed in hippocampal neurons and glia. (A) untransfected rat hippocampal microcultures were stained at 10 DIV with an antibody that selectively reacts with truncated TrkB receptors (TrkB[TK–]). (B) As a positive control, rat hippocampal neurons were transfected at 9 DIV with wt TrkB.T1 receptor and also processed for TrkB [TK–] immunocytochemistry. Neurons were identified by co-staining with a MAP2 antibody. Truncated TrkB receptors can be immunostained in untransfected neurons (arrows) and glia (asterisk). Specificity of the TrkB[TK–] antibody was evident from the intense signal in TrkB.T1-overexpressing neurons in B. Owing to the tenfold shorter exposure time for the overexpressed truncated receptor staining in the glia is not visible in B (lower panel). Scale bar: 10 μm.

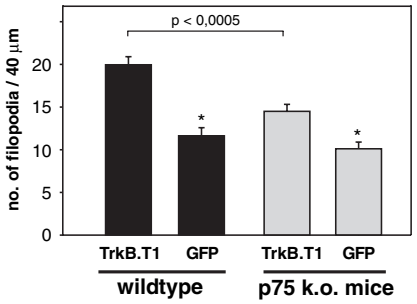

Fig. 9. Reduction of TrkB.T1-induced filopodia formation in hippocampal neurons from p75 knockout mice. Neurons from wt or $p75^{NTR}$ knockout mice were transfected with either TrkB.T1-GFP or GFP. The reduction in the density of TrkB.T1-induced filopodia in $p75^{NTR}$ knockout compared with wild-type mice is highly significant ($P<0.0005$). Both TrkB.T1-transfected groups were also significantly different from the respective GFP-transfected negative controls (*$P<0.0001$). The number of filopodia in GFP-expressing cells from $p75^{NTR}$ knockout mice was not significantly different from GFP-expressing wild-type cultures ($P>0.15$) (n=28-52 cells from two to three independent experiments).

In trying to rescue the inhibited filopodial growth in the exon III p75^{NTR} knockout mice, we cotransfected one group of the knockout-derived cells with TrkB.T1-GFP and the wt-p75^{NTR} receptor, respectively. However, as previously observed in rat neurons (see above) overexpression of wt-p75^{NTR} also induced the death of mice neurons, thus impairing the observation of a rescue effect.

Discussion

Our results indicate a novel signalling mechanism of truncated TrkB receptors in regulating dendritic growth of hippocampal neurons. Overexpression of truncated TrkB receptors (TrkB.T1 and TrkB.T2) induced a twofold increase in the number of dendritic filopodia. This effect was independent of the presence of neurotrophins and of tyrosine kinase signalling of endogenous TrkB.FL. Our data suggest an extracellular or intramembrane interaction between TrkB.T1 and p75^{NTR} receptors, initiating filopodial growth via p75^{NTR} intracellular signalling. This model is corroborated by the dominant negative regulation of the TrkB.T1-induced filopodia by coexpression of a p75^{NTR} lacking the intracellular domain (p75ΔICD) and by the significant reduction of the TrkB.T1 effect in p75^{NTR} knockout mice. Coexpressing surplus TrkB.FL receptors antagonized the TrkB.T1-induced filopodial growth independently of Trk kinase activity, suggesting a competitive interference of TrkB.FL in the formation of putative p75^{NTR}-TrkB.T1 heterodimers.

Since overexpression of the dominant negative p75ΔICD was able to also reduce basal levels of filopodia in our hippocampal cultures (Fig. 7D), these data suggest a role of endogenous TrkB.T1-p75^{NTR} signalling in dendritic growth. Accordingly TrkB.T1 was observed to be expressed endogeneously in our hippocampal neurons (Fig. 8).

The absence of a similar inhibition of basal levels of filopodia upon overexpression of TrkB.FL (Fig. 4), might reflect the different potencies of these two dominant negative inhibitors. In this context it is of note that p75ΔICD was able to inhibit TrkB.T1-GFP-induced filopodia when coexpressed at a DNA ratio of only 1:3 (Fig. 7) whereas TrkB.FL needed to be overexpressed at a DNA ratio of 3:1 (Fig. 5) in order to have a similar dominant negative effect.

The unperturbed presence of filopodia in GFP-expressing controls from exon III p75^{NTR} knockout mice might be accounted for by the expression of the p75^{NTR} short in these neurons, which can partially substitute for the wt p75^{NTR}. In addition, it seems conceivable that TrkB.T1-p75^{NTR}-mediated induction of filopodia is not the sole mechanism inducing dendritic growth in hippocampal neurons, thus leaving space for induction of filopodia in the p75 knockout mice via other pathways.

Previous studies showed that cell surface targeting of TrkB.FL in hippocampal neurons and in retinal ganglion cells is promoted by electrical activity and decreased by coexpression of TrkB.T1 (Meyer-Franke et al., 1998; Du et al., 2000; Haapasalo et al., 2002). Since we observed cell surface targeting of TrkB.FL and of all other TrkB receptors tested, and given the comparable surface targeting of GFP-tagged and untagged TrkB.FL (Klau et al., 2001), these data suggest that sufficient levels of electrical activity are present in our neurons to support this targeting process, both in the absence and presence of C-terminal GFP. Interestingly, membrane delimited fluorescence of TrkB.FL-GFP was decreased upon action potential blockade with TTX

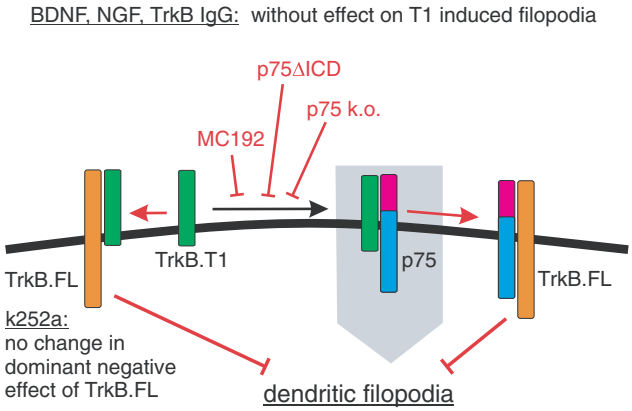

Fig. 10. Proposed model of TrkB.T1 action in the induction of filopodia. Interaction of TrkB.T1 with the p75^{NTR}, leads to the formation of filopodia (grey arrow). This effect can be blocked by either the MC192 antibody or the dominant negative p75ΔICD (inhibitory agents in red). The dominant negative action of the TrkB.FL receptor can be explained by its competition with TrkB.T1 for binding to either p75^{NTR} (right margin) or by scavenging TrkB.T1 (left margin). NGF, BDNF, TrkB-IgG or K252a were without effect on this signalling mechanism. The short p75^{NTR} (in blue) indicates a possible (albeit weaker) such signalling via a p75^{NTR} lacking an intact extracellular domain (see p75^{NTR} knockout mice experiments)

(M.H. and V.L., unpublished results), further supporting the view that activity-dependent targeting of TrkB.FL-GFP is intact in our neurons.

We can not definitely exclude that overexpression of TrkB.T1 decreases surface expression of endogenous TrkB.FL in our neurons (Haapasalo et al., 2002). However, since induction of filopodia by TrkB.T1 occurs independently from ligand binding, it would remain unclear how reduced endogenous TrkB.FL levels could increase the number of filopodia, if not by the mechanism suggested in Fig. 10: given the dominant negative effect of coexpressed TrkB.FL on TrkB.T1-induced filopodia (Fig. 5), reduced surface levels of endogenous TrkB.FL should facilitate the growth of filopodia, thus being in accordance with our model (see Fig. 10).

TrkB.T1-induced morphological changes

A previous study by Yacoubian and (Lo Yacoubian and Lo, 2000) in ferret cortical slices suggested a role of overexpressed TrkB.T1 receptors in promoting the growth of distal dendrites, whereas TrkB.FL induced the growth of proximal dendrites. Exogenous TrkB ligands reversed the effect of TrkB.T1 in a kinase-dependent manner, whereas TrkB.FL effects were potentiated. Although this was an intriguing finding, possible downstream effectors of this TrkB.T1-induced growth remained elusive. Their results were most compatible with a mutual regulation of dendritic growth by both receptor isoforms: high local expression ratios of TrkB.FL:TrkB.T1 would favour a ligand-dependent growth of dendrites, whereas the relative abundance of TrkB.T1 would promote dendritic growth in the absence of ligands.

In our hippocampal cultures, the basic finding of TrkB.T1-induced growth of dendritic filopodia is comparable with the TrkB.T1-induced dendritic branching observed by Yacoubian

and Lo (Yacoubian and Lo, 2000). However, two differences are evident. First, in our study, the proximal dendrites were the site of filopodia formation. Secondly, all TrkB.T1-induced effects on the formation of filopodia in our hippocampal cultures were completely independent of endogenous and exogenous TrkB ligands, and of TrkB.FL kinase signalling (Fig. 4). It remains an attractive hypothesis, that subcellular differences in endogenous expression levels of TrkB.FL versus TrkB.T1 (e.g. in hippocampus versus neocortex) could account for this dissimilar ligand dependence in TrkB-dependent dendritic growth (Yacoubian and Lo, 2000).

We directly addressed this issue by coexpressing both receptor isoforms in the same cells (Fig. 5). The observed dominant negative effect of TrkB.FL on TrkB.T1-induced formation of dendritic filopodia is the first direct demonstration of full length TrkB-mediated inhibition of TrkB.T1-induced signalling in neurons. Interestingly, a comparable dominant negative regulation of TrkB.T1-induced morphological changes by TrkB.FL receptors has recently been shown in a neuroblastoma cell line (Haapasalo et al., 1999). Taken together, these data provide evidence for a new function of coordinated TrkB.T1 and TrkB.FL signalling in regulating dendritic growth.

In contrast to the effects on filopodial growth observed here, most previous studies, addressing TrkB-dependent modulation of dendritic growth, focussed on BDNF-dependent modulation of dendritic branching and dendritic length (McAllister et al., 1997; Lom and Cohen-Cory, 1999; Tolwani et al., 2002). However, Horch and colleagues (Horch et al., 1999) reported a BDNF overexpression-induced sprouting of dendritic protrusions in cortical neurons, similar to the TrkB.T1-induced (albeit ligand independent) filopodial growth in our hippocampal neurons. The relation between these two effects remains to be determined.

Given the delayed expression of TrkB.T1 receptors during development compared with TrkB.FL (Fryer et al., 1996), it seems to be an attractive hypothesis that BDNF-dependent signalling via full length receptors supports the outgrowth of primary dendrites early in the development, whereas the delayed expression of TrkB.T1 allows for the ligand-independent sprouting of dendritic filopodia during synapse formation (Yacoubian and Lo, 2000). In a preliminary set of experiments we determined whether the increased number of dendritic filopodia is accompanied by an increase in synaptic contacts as suggested previously (Ziv and Smith, 1996). However, activity-dependent staining of presynaptic terminals using FM 4-64 did not indicate a rise in functional synapse numbers in our TrkB.T1-overexpressing neurons (M.H. and V.L., unpublished observation) (see also Klau et al., 2001). It remains to be established whether such an effect on synaptic differentiation will be detected in TrkB.T1-GFP-overexpressing neurons in hippocampal slice cultures that show a larger number of coherent afferent fibres innervating the dendritic tree of a given neuron.

Signalling mechanism of TrkB.T1-induced growth of dendritic filopodia

Our data provide three independent lines of evidence for an involvement of the $p75^{NTR}$ in the observed TrkB.T1-induced formation of filopodia: (1) inhibition in the presence of the MC 192 monoclonal $p75^{NTR}$ antibody; (2) dominant negative inhibition by a $p75^{NTR}$ lacking the intracellular domain; and, (3) significant reduction of the effect in exon III $p75^{NTR}$ knockout mice.

Furthermore, growth of filopodia is intact upon overexpression of the mutant TrkBΔICD receptor variant lacking the TrkB.T1 intracellular domain. Coexpression of TrkB.FL receptors inhibits TrkB.T1-induced filopodia in a dominant negative fashion.

Taken together, these results lead us to propose that ligand-independent interaction of the extracellular or the intramembrane domains of TrkB.T1 and $p75^{NTR}$ provokes the formation of dendritic filopodia (Fig. 10). This pathway can be blocked by sterical hindrance of the TrkB.T1-$P75^{NTR}$ interaction by extracellular binding of the MC192 antibody to the $p75^{NTR}$ receptor (Barker and Shooter, 1994; Maliartchouk and Saragovi, 1997; Kimpinski et al., 1999). The inability of a different $p75^{NTR}$ antibody to block a similar TrkB.T1-induced growth of filopodia in N2a cells (Haapasalo et al., 1999) might reflect the different specificities of these antibodies to interfere with the interaction of $p75^{NTR}$ and TrkB.T1.

In accordance with the model, the filopodial growth is blocked when the TrkB.T1-$p75^{NTR}$ interaction is inhibited by a dominant negative $p75^{NTR}$ (Fig. 7). Furthermore we suggest that TrkB.FL inhibits the binding of TrkB.T1 to the $p75^{NTR}$ receptor by either forming heterodimers with TrkB.T1 or with $p75^{NTR}$, respectively, thereby inhibiting the specific downstream signalling of the putative TrkB.T1-$p75^{NTR}$ heterodimers. Thus, the tendency of TrkB.T1 to induce growth of filopodia in a given dendritic segment would critically depend on the relative abundance of these three receptors at the specified subcellular location (see also Yacoubian and Lo, 2000).

Interestingly, the TrkB.T1 receptor, which lacks an intracellular domain (TrkBΔICD), still induced filopodia (Fig. 4C), indicating that the required motifs for interaction with the $p75^{NTR}$ are localized in the extracellular or intramembrane domain of TrkB.T1. Although these domains are identical in TrkB.FL, this receptor lacked an effect on filopodia. These data suggest that the intracellular domain of TrkB.FL prevents the appropriate interaction of the extracellular or intramembrane domain of the TrkB receptor with the $p75^{NTR}$, or hinders the contact of a putative TrkB.FL-$p75^{NTR}$ complex with the intracellular filopodia inducing machinery. Similarly, the dominant negative effect of coexpressed TrkB.FL on TrkB.T1-induced filopodia (Fig. 5) could be accounted for by this mechanism. Consequently, future studies should aim at identifying the TrkB.FL intracellular domains that prevent TrkB.FL from inducing filopodia.

The significant reduction of the TrkB.T1-induced filopodia in p75 knockout cultures supports the model proposed in Fig. 10. However, the exon III p75 knockout mouse used in this study has been reported previously to give rise to expression of a short $p75^{NTR}$ lacking most of the extracellular domain, which can partially substitute for full length $p75^{NTR}$ receptor signalling (von Schack et al., 2001). Thus, the remaining effect on filopodia formation in our p75 knockout experiments (see Fig. 9) could be explained by intact signalling of this short $p75^{NTR}$. In fact recent evidence directly suggests signalling of $p75^{NTR}$ lacking an extracellular domain, which results from intramembrane proteolytic processing of the $p75^{NTR}$ (Yamashita et al., 1999; Jung et al., 2003; Kanning et al., 2003; Yamashita and Tohyama, 2003).

In accordance with the model in Fig. 10 recent evidence in

fact indicates a ligand-independent molecular interaction of both TrkB.FL and intracellularly truncated TrkB.FL (resembling TrkB.T1) with the p75^{NTR} receptor in HEK A293 cells, and also shows a role of these heteromeric receptor complexes in ligand discrimination (Bibel et al., 1999). Similar results have been described with respect to a molecular interaction of TrkA with p75^{NTR} (Ross et al., 1996; Gargano et al., 1997) and a functional interaction of TrkC receptors with p75^{NTR} (Hapner et al., 1998) in non-neuronal cells. Our data now provide the first evidence for a possible physiological function of a TrkB-p75^{NTR} heteromeric receptor in neurons (see above).

NGF, which is known to selectively activate p75^{NTR} and to induce fibre outgrowth via ceramide production in hippocampal neurons (Brann et al., 1999; Friedman, 2000), did not mimic the effect of overexpressed TrkB.T1 in our cultures. The involvement of different p75^{NTR}-dependent signalling cascades in response to TrkB.T1 versus NGF could account for the dissimilar effects of these two unlike ligands in stimulating p75^{NTR}-induced dendritic growth. The p75^{NTR}-mediated dendritic growth in hippocampal neurons following stimulation with NGF was restricted to very young embryonic cultures (Brann et al., 1999; Brann et al., 2002). In more mature neurons, NGF-induced effects on dendritic branching were absent although the expression level of p75 receptors was not decreased (Brann, 2002). It thus seems plausible to assume that p75^{NTR} signalling is experiencing subtle changes during neuronal development in culture, which can not be explained by, for example, reduced expression levels of p75^{NTR} protein. The ongoing expression of p75^{NTR} as observed by Brann et al. (Brann et al., 2002) is in line with stable expression of p75 mRNA in our hippocampal microcultures until at least 14 DIV (M. Knipper and V. L., unpublished). In support of a role of p75^{NTR} in the formation of neuronal processes, Yamashita and colleagues (Yamashita et al., 1999) identified the small G protein RhoA as interacting with the intracellular domain of p75^{NTR}. This interaction leads to a neurotrophin-independent activation of RhoA, indicating ligand-independent signalling of the p75 receptors. Binding of neurotrophins to p75^{NTR} reduces the activity of RhoA, resulting in axonal outgrowth in PNS neurons (Yamashita et al., 1999). Interestingly, Rho has recently also been demonstrated to regulate the growth of filopodia and spine formation in hippocampal dendrites (Nakayama et al., 2000). It is thus tempting to speculate that RhoA signalling is involved in the TrkB.T1-p75^{NTR}-mediated growth of filopodia observed in our study.

Myelin-associated glycoprotein (MAG) was recently found to bind to a complex consisting of p75^{NTR} and ganglioside GT1b, thus retarding neurite outgrowth via intermediate activation of Rho (Yamashita et al., 2002), whereas neurotrophin-stimulated p75^{NTR} promotes neurite outgrowth. Likewise, two recent studies implicated the p75^{NTR} in the signal transduction of the NoGo receptor in complex with its ligands Nogo66, nMAG and oMPG (Wang et al., 2002; Wong et al., 2002). These findings are particularly relevant to our results, stressing the binding of p75^{NTR} receptors to ligands other than neurotrophins. Furthermore, in the study by Yamashita and colleagues (Yamashita et al., 2002) MAG was shown to stimulate Rho activity downstream of p75^{NTR}, whereas activation via neurotrophins elicits an opposite p75^{NTR} signalling mechanism. This suggests that p75^{NTR}-induced RhoA signalling could also be involved in the TrkB.T1-induced outgrowth of filopodia in our hippocampal neurons. Since manipulation of Rho activity induces marked changes in dendritic morphology on its own (Nakayama et al., 2000), a role of Rho in TrkB.T1-induced growth of filopodia can not be easily determined by inhibition of Rho function. Thus, future studies employing mutant p75 receptors lacking the Rho interacting mastoparan binding site could help to demonstrate a causal connection between Rho binding to p75 and TrkB.T1-induced formation of filopodia in hippocampal neurons.

The authors want to thank David Middlemas for providing rat TrkB constructs, Sabine Rickheim-Lowack and Sabine Laerbusch for excellent technical assistance and Heiko Luhmann and Rolf Heumann for their support. This work was supported by grants from the DFG to V.L. (SFB 509 and SFB 553), M.S. (SFB 487) and K.S.E. (SFB 452).

References

Barker, P. A. and Shooter, E. M. (1994). Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. *Neuron* **13**, 203-215.

Baxter, G. T., Radeke, M. J., Kuo, R. C., Makrides, V., Hinkle, B., Hoang, R., Medina-Selby, A., Coit, D., Valenzuela, P. and Feinstein, S. C. (1997). Signal transduction mediated by the truncated trkB receptor isoforms, trkB.T1 and trkB.T2. *J. Neurosci.* **17**, 2683-2690.

Bibel, M., Hoppe, E. and Barde, Y. A. (1999). Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. *EMBO J.* **18**, 616-622.

Biffo, S., Offenhauser, N., Carter, B. D. and Barde, Y. A. (1995). Selective binding and internalisation by truncated receptors restrict the availability of BDNF during development. *Development* **121**, 2461-2470.

Bothwell, M. (1995). Functional interactions of neurotrophins and neurotrophin receptors. *Annu. Rev. Neurosci.* **18**, 223-253.

Brann, A. B., Scott, R., Neuberger, Y., Abulafia, D., Boldin, S., Fainzilber, M. and Futerman, A. H. (1999). Ceramide signaling downstream of the p75 neurotrophin receptor mediates the effects of nerve growth factor on outgrowth of cultured hippocampal neurons. *J. Neurosci.* **19**, 8199-8206.

Brann, A. B., Tcherpakov, M., Williams, I. M., Futerman, A. H. and Fainzilber, M. (2002). Nerve growth factor-induced p75-mediated death of cultured hippocampal neurons is age-dependent and transduced through ceramide generated by neutral sphingomyelinase. *J. Biol. Chem.* **277**, 9812-9818.

Cabelli, R. J., Allendoerfer, K. L., Radeke, M. J., Welcher, A. A., Feinstein, S. C. and Shatz, C. J. (1996). Changing patterns of expression and subcellular localization of TrkB in the developing visual system. *J. Neurosci.* **16**, 7965-7980.

Dobrowsky, R. T. and Carter, B. D. (1998). Coupling of the p75 neurotrophin receptor to sphingolipid signaling. *Ann. N. Y. Acad. Sci.* **845**, 32-45.

Du, J., Feng, L., Yang, F. and Lu, B. (2000). Activity- and Ca(2+)-dependent modulation of surface expression of brain-derived neurotrophic factor receptors in hippocampal neurons. *J. Cell Biol.* **150**, 1423-1434.

Eide, F. F., Vining, E. R., Eide, B. L., Zang, K., Wang, X. Y. and Reichardt, L. F. (1996). Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling. *J. Neurosci.* **16**, 3123-3129.

Fanger, G. R., Jones, J. R. and Maue, R. A. (1995). Differential regulation of neuronal sodium channel expression by endogenous and exogenous tyrosine kinase receptors expressed in rat pheochromocytoma cells. *J. Neurosci.* **15**, 202-213.

Friedman, W. J. (2000). Neurotrophins induce death of hippocampal neurons via the p75 receptor. *J. Neurosci.* **20**, 6340-6346.

Fryer, R. H., Kaplan, D. R., Feinstein, S. C., Radeke, M. J., Grayson, D. R. and Kromer, L. F. (1996). Developmental and mature expression of full-length and truncated TrkB receptors in the rat forebrain. *J. Comp Neurol.* **374**, 21-40.

Gargano, N., Levi, A. and Alema, S. (1997). Modulation of nerve growth factor internalization by direct interaction between p75 and TrkA receptors. *J. Neurosci. Res.* **50**, 1-12.

Haapasalo, A., Saarelainen, T., Moshnyakov, M., Arumae, U., Kiema, T. R., Saarma, M., Wong, G. and Castren, E. (1999). Expression of the naturally occurring truncated trkB neurotrophin receptor induces outgrowth of filopodia and processes in neuroblastoma cells. *Oncogene* **18**, 1285-1296.

Haapasalo, A., Sipola, I., Larsson, K., Akerman, K. E., Stoilov, P., Stamm, S., Wong, G. and Castren, E. (2002). Regulation of TRKB surface expression by brain-derived neurotrophic factor and truncated TRKB isoforms. *J. Biol Chem.* **277**, 43160-43167.

Hapner, S. J., Boeshore, K. L., Large, T. H. and Lefcort, F. (1998). Neural differentiation promoted by truncated trkC receptors in collaboration with p75(NTR). *Dev. Biol.* **201**, 90-100.

Harris, K. M. (1999). Structure, development, and plasticity of dendritic spines. *Curr. Opin. Neurobiol.* **9**, 343-348.

Hartmann, M., Heumann, R. and Lessmann, V. (2001). Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. *EMBO J.* **20**, 5887-5897.

Haubensak, W., Narz, F., Heumann, R. and Lessmann, V. (1998). BDNF-GFP containing secretory granules are localized in the vicinity of synaptic junctions of cultured cortical neurons. *J. Cell Sci.* **111**, 1483-1493.

Horch, H. W., Kruttgen, A., Portbury, S. D. and Katz, L. C. (1999). Destabilization of cortical dendrites and spines by BDNF. *Neuron* **23**, 353-364.

Ip, N. Y., Li, Y., Yancopoulos, G. D. and Lindsay, R. M. (1993a). Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. *J. Neurosci.* **13**, 3394-3405.

Ip, N. Y., Stitt, T. N., Tapley, P., Klein, R., Glass, D. J., Fandl, J., Greene, L. A., Barbacid, M. and Yancopoulos, G. D. (1993b). Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. *Neuron* **10**, 137-149.

Jung, K. M., Tan, S., Landman, N., Petrova, K., Murray, S., Lewis, R., Kim, P. K., Kim, D. S., Ryu, S. H., Chao, M. V. and Kim, T. W. (2003). Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor. *J. Biol Chem.* **278**, 42161-42169.

Kanning, K. C., Hudson, M., Amieux, P. S., Wiley, J. C., Bothwell, M. and Schecterson, L. C. (2003). Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. *J. Neurosci.* **23**, 5425-5436.

Kaplan, D. R. and Miller, F. D. (2000). Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* **10**, 381-391.

Kimpinski, K., Jelinski, S. and Mearow, K. (1999). The anti-p75 antibody, MC192, and brain-derived neurotrophic factor inhibit nerve growth factor-dependent neurite growth from adult sensory neurons. *Neuroscience* **93**, 253-263.

Klau, M., Hartmann, M., Erdmann, K. S., Heumann, R. and Lessmann, V. (2001). Reduced number of functional glutamatergic synapses in hippocampal neurons overexpressing full-length TrkB receptors. *J. Neurosci. Res.* **66**, 327-336.

Klein, R., Conway, D., Parada, L. F. and Barbacid, M. (1990). The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell* **61**, 647-656.

Kryl, D. and Barker, P. A. (2000). TTIP is a novel protein that interacts with the truncated T1 TrkB neurotrophin receptor. *Biochem. Biophys. Res. Commun.* **279**, 925-930.

Kryl, D., Yacoubian, T., Haapasalo, A., Castren, E., Lo, D. and Barker, P. A. (1999). Subcellular localization of full-length and truncated Trk receptor isoforms in polarized neurons and epithelial cells. *J. Neurosci.* **19**, 5823-5833.

Lambert, R. C., Maulet, Y., Dupont, J. L., Mykita, S., Craig, P., Volsen, S. and Feltz, A. (1996). Polyethylenimine-mediated DNA transfection of peripheral and central neurons in primary culture: probing Ca^{2+} channel structure and function with antisense oligonucleotides. *Mol. Cell Neurosci.* **7**, 239-246.

Lee, K. F., Li, E., Huber, L. J., Landis, S. C., Sharpe, A. H., Chao, M. V. and Jaenisch, R. (1992). Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell* **69**, 737-749.

Lessmann, V. and Heumann, R. (1998). Modulation of unitary glutamatergic synapses by neurotrophin-4/5 or brain-derived neurotrophic factor in hippocampal microcultures: presynaptic enhancement depends on pre-established paired-pulse facilitation. *Neuroscience* **86**, 399-413.

Levi-Montalcini, R. and Hamburger, V. (1953). A diffusible agent of mouse sarcoma, producing hyperplasia of sympathetic-ganglia and hyperneurotization of viscera in the chick embryo. *J. Exp. Zool.* **123**, 233-287.

Lom, B. and Cohen-Cory, S. (1999). Brain-derived neurotrophic factor differentially regulates retinal ganglion cell dendritic and axonal arborization in vivo. *J. Neurosci.* **19**, 9928-9938.

Lu, B. (2003). BDNF and activity-dependent synaptic modulation. *Learn. Mem.* **10**, 86-98.

Majdan, M., Lachance, C., Gloster, A., Aloyz, R., Zeindler, C., Bamji, S., Bhakar, A., Belliveau, D., Fawcett, J., Miller, F. D. and Barker, P. A. (1997). Transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor undergo neuronal apoptosis. *J. Neurosci.* **17**, 6988-6998.

Maliartchouk, S. and Saragovi, H. U. (1997). Optimal nerve growth factor trophic signals mediated by synergy of TrkA and p75 receptor-specific ligands. *J. Neurosci.* **17**, 6031-6037.

McAllister, A. K., Katz, L. C. and Lo, D. C. (1997). Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. *Neuron* **18**, 767-778.

Meyer-Franke, A., Wilkinson, G. A., Kruttgen, A., Hu, M., Munro, E., Hanson, M. G., Jr, Reichardt, L. F. and Barres, B. A. (1998). Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. *Neuron* **21**, 681-693.

Middlemas, D. S., Lindberg, R. A. and Hunter, T. (1991). trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. *Mol. Cell. Biol.* **11**, 143-153.

Nakayama, A. Y., Harms, M. B. and Luo, L. (2000). Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. *J. Neurosci.* **20**, 5329-5338.

Ninkina, N., Adu, J., Fischer, A., Pinon, L. G., Buchman, V. L. and Davies, A. M. (1996). Expression and function of TrkB variants in developing sensory neurons. *EMBO J.* **15**, 6385-6393.

Patapoutian, A. and Reichardt, L. F. (2001). Trk receptors: mediators of neurotrophin action. *Curr. Opin. Neurobiol.* **11**, 272-280.

Poo, M. M. (2001). Neurotrophins as synaptic modulators. *Nat. Rev. Neurosci.* **2**, 24-32.

Rose, C. R., Blum, R., Pichler, B., Lepier, A., Kafitz, K. W. and Konnerth, A. (2003). Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. *Nature* **426**, 74-78.

Ross, A. H., Daou, M. C., McKinnon, C. A., Condon, P. J., Lachyankar, M. B., Stephens, R. M., Kaplan, D. R. and Wolf, D. E. (1996). The neurotrophin receptor, gp75, forms a complex with the receptor tyrosine kinase TrkA. *J. Cell Biol.* **132**, 945-953.

Roux, P. and Barker, P. (2002). Neurotrophin signaling through the p75 neurotrophin receptor. *Prog. Neurobiol.* **67**, 203.

Shelton, D. L., Sutherland, J., Gripp, J., Camerato, T., Armanini, M. P., Phillips, H. S., Carroll, K., Spencer, S. D. and Levinson, A. D. (1995). Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. *J. Neurosci.* **15**, 477-491.

Tolwani, R., Buckmaster, P., Varma, S., Cosgaya, J., Wu, Y., Suri, C. and Shooter, E. (2002). BDNF overexpression increases dendrite complexity in hippocampal dentate gyrus. *Neuroscience* **114**, 795.

Von Schack, D., Casademunt, E., Schweigreiter, R., Meyer, M., Bibel, M. and Dechant, G. (2001). Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and the vascular system. *Nat. Neurosci.* **4**, 977-978.

Wang, K. C., Kim, J. A., Sivasankaran, R., Segal, R. and He, Z. (2002). p75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature* **420**, 74-78.

Watson, F. L., Heerssen, H. M., Moheban, D. B., Lin, M. Z., Sauvageot, C. M., Bhattacharyya, A., Pomeroy, S. L. and Segal, R. A. (1999). Rapid nuclear responses to target-derived neurotrophins require retrograde transport of ligand-receptor complex. *J. Neurosci.* **19**, 7889-7900.

Wong, S. T., Henley, J. R., Kanning, K. C., Huang, K. H., Bothwell, M. and Poo, M. M. (2002). A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. *Nat. Neurosci.* **5**, 1302-1308.

Yacoubian, T. A. and Lo, D. C. (2000). Truncated and full-length TrkB receptors regulate distinct modes of dendritic growth. *Nat. Neurosci.* **3**, 342-349.

Yamashita, T. and Tohyama, M. (2003). The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. *Nat. Neurosci.* **6**, 461-467.

Yamashita, T., Tucker, K. L. and Barde, Y. A. (1999). Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron* **24**, 585-593.

Yamashita, T., Higuchi, H. and Tohyama, M. (2002). The p75 receptor transduces the signal from myelin-associated glycoprotein to Rho. *J. Cell Biol.* **157**, 565-570.

Ziv, N. E. and Smith, S. J. (1996). Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* **17**, 91-102.