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Signal Transduction Events Mediated by the BDNF Receptor gp145^{trkB} in Primary Hippocampal Pyramidal Cell Culture

H. Nicholas Marsh,[1] Wendy K. Scholz,[1] Fabienne Lamballe,[2] Rüdiger Klein,[2] Venkata Nanduri,[2] Mariano Barbacid,[2] and H. Clive Palfrey[1]

[1]Department of Pharmacological and Physiological Sciences, University of Chicago, Chicago, Illinois 60637 and [2]Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543

The *trk*B gene encodes a tyrosine kinase receptor, gp145^{trkB}, for brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4). To understand the role of gp145^{trkB} in the nervous system, we have investigated its expression in embryonic rat hippocampal pyramidal cell cultures and examined the effects of BDNF on signal transduction in the primary neurons. The expression of *trk*B transcripts was established by PCR analysis and *in situ* hybridization. In addition to gp145^{trkB}, the pyramidal neuronal cultures expressed transcripts specific for the NT-3 receptor gp145^{trkC}, but not for the high-affinity NGF receptor gp140^{trk} or for p75^{LNGFR}, a low-affinity receptor for all known members of the NGF family of neurotrophins including the gp145^{trkB} ligands, BDNF and NT-4. The presence of gp145^{trkB} receptors in the primary neuronal cultures was confirmed by immunocytochemical analysis in which >90% of the cells stained with affinity-purified polyclonal antibodies to gp145^{trkB}. Immunoblots using this antibody revealed a single ~140 kDa protein in both adult hippocampus and pyramidal cultures. Addition of recombinant BDNF to these cultures induced the tyrosine phosphorylation of gp145^{trkB}, as determined by anti-phosphotyrosine staining of gp145^{trkB} immunoprecipitates. Moreover, BDNF treatment activated the microtubule-associated protein (MAP) kinases, as determined by an increase in MAP2 phosphorylation *in vitro*. Both the 41 and 44 kDa forms of MAP kinase were activated by BDNF. BDNF also increased c-*fos* expression in over 90% of the cells. These results indicate that gp145^{trkB} does not require p75^{LNGFR} to form a functional receptor for BDNF in hippocampal pyramidal neurons.

[Key words: low-affinity NGF receptor, receptor tyrosine kinase, protein tyrosine phosphorylation, MAP kinase activation, c-Fos induction]

NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) comprise a family of neurotrophic factors necessary for the development and maintenance of specific neurons (for review, see Thoenen, 1991). Though these neurotrophins share roughly 60% amino acid homology, each has a very distinct range of biological activities. NGF is essential for the growth of sympathetic and septal cholinergic neurons, as well as sensory ganglion cells derived from the neural crest (Levi-Montalcini, 1987). BDNF, initially isolated from pig brain (Barde et al., 1982), also supports the survival of these types of neurons (Alderson et al., 1990) but, in addition, acts on dopaminergic neurons of the substantia nigra, GABAergic neurons of the basal forebrain, and rat retinal ganglion cells (Johnson et al., 1986; Hyman et al., 1991; Knüsel et al., 1991). NT-3, on the other hand, exhibits trophic effects only on neural crest– and nodose ganglion–derived sensory neurons (Maisonpiérre et al., 1990; Knüsel et al., 1991). NT-4, originally discovered in *Xenopus* ovary (Hallböök et al., 1991) and subsequently found in mammals (Berkemeier et al., 1991; Ip et al., 1992), has been recently shown to promote the survival of peripheral sensory and sympathetic neurons (Berkemeier et al., 1991).

Together with a distinct range of biological activities, each neurotrophin displays a unique pattern of distribution. The highest levels of NGF mRNA have been found in the hippocampus and cerebral cortex, along with other areas receiving major cholinergic projections from the basal forebrain (Shelton and Reichardt, 1986). BDNF mRNA appears to be more widespread in the brain than mRNA for either NGF or NT-3, but like NGF, BDNF is most abundant in the hippocampus, cortex, and other synaptic targets of basal forebrain cholinergic neurons (Ernfors et al., 1990; Phillips et al., 1990). In fact, the level of mRNA for BDNF is approximately 50 times higher than NGF mRNA in the hippocampus (Hofer et al., 1990).

It is generally accepted that the NGF family of neurotrophins achieve their effects by binding to cell surface receptors that initiate a series of intracellular events culminating in phenotypic changes that are cell type specific. In the case of NGF, the neurotrophin binds to two distinct receptor types: low-affinity receptors (LNGFR; $K_d \approx 10^{-9}$ M) and high-affinity receptors (HNGFR; $K_d \approx 10^{-11}$ M) (for review, see Misko et al., 1987). It is, however, generally acknowledged that HNGFR must play a central role in mediating the physiological actions of the NGF family of neurotrophins. Several groups have demonstrated that the products of the Trk family of tyrosine protein kinases (see Barbacid et al., 1991, for review) are also receptors for these neurotrophins (Hempstead et al., 1991; Kaplan et al., 1991a; Klein et al., 1991a,b; Lamballe et al., 1991; Soppet et al., 1991; Squinto et al., 1991). These receptors fulfill several criteria thought to be necessary for biological function. For example, they exhibit specificity for each neurotrophin. In addition, they mediate full mitogenic and survival responses when ectopically expressed in cells lacking p75^{LNGFR} and can mediate the differ-

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Correspondence should be addressed to H. Clive Palfrey, Ph.D., Department of Pharmacological and Physiological Sciences, The University of Chicago, 947 East 58th Street, Chicago, IL 60637.

entiation of PC12 cells in response to their cognate ligands (see Barbacid, 1993, for review). Other investigators have proposed, based on direct receptor binding studies, that high-affinity neurotrophin receptors and particularly the HNGFR are heterodimers of the low-affinity $p75^{LNGFR}$ receptor and respective members of the Trk family of tyrosine kinases (Hempstead et al., 1991; Kaplan et al., 1991b). However, these results are at variance with those of Barbacid and coworkers, who have reported high-affinity binding of NGF and NT-3 to their cognate $gp140^{trk}$ and $gp145^{trkC}$ receptors in the absence of $p75^{LNGFR}$ (Klein et al., 1991a; Lamballe et al., 1991; Jing et al., 1992).

The mRNAs coding for the Trk receptors appear to have a primarily neuronal localization as determined by *in situ* hybridization studies (Klein et al., 1989, 1990; Martin-Zanca et al., 1989; Lamballe et al., 1991). *trk* mRNA can be detected in dorsal root ganglia as well as in cranial sensory ganglia, including the trigeminal, superior, and jugular ganglia in the adult brain (Martin-Zanca et al., 1989). *trk*B transcripts can be found in multiple structures of the CNS such as the cortical layers, the choroid plexus, the lining of certain ventricles, the cerebral cortex, the thalamus, and the pyramidal cell layer of the hippocampus as well as in the PNS (Klein et al., 1989, 1990). Similarly, *trk*C is not only expressed in the pyramidal cell layer of the hippocampus and the cerebral cortex, but is also found in the granular layer of the cerebellum (Lamballe et al., 1991) and in multiple structures of the PNS (Lamballe et al., 1993).

Thus far, the study of signal transduction events in response to neurotrophins has largely been confined to continuous cell lines or non-neuronal cells transfected with the appropriate receptors. Consequently, the mechanism of action of the neurotrophins in CNS neurons is entirely unknown. The detection of *trk*B transcripts in the pyramidal layer of the hippocampus by *in situ* hybridization (Klein et al., 1989, 1990) prompted us to investigate the possibility that these neuronal cells might be a useful model to study the signal transduction events initiated by BDNF and to resolve the relative contributions of the $p75^{LNGFR}$ and TrkB receptors in mediating these effects.

A preliminary account of this work has been presented (Marsh et al., 1992).

Materials and Methods

Hippocampal pyramidal neuron cultures. Astrocytes were prepared from the cerebral hemispheres of newborn rats by the procedure of Booher and Sensenbrenner (1972). After 4 d in culture, glial cells were removed from the dishes with trypsin and plated on 30 mm Thermanox coverslips equipped with Paraplast "feet." Pyramidal neurons were prepared from the hippocampi of fetal rats of 17 d of gestation (E17) as described by Bartlett and Banker (1984) with some modifications (Scholz and Palfrey, 1991). Hippocampi were dissected in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS) and incubated in 0.1% trypsin (Worthington) for 20 min. Tissue was dissociated by triturating one to seven passes with a normal-bore Pasteur pipette and 7–10 times with a flame-narrowed Pasteur pipette. Cells were plated in Dulbecco's modified Eagle's medium (Hazelton) plus 10% horse serum (GIBCO) on poly-L-lysine (Sigma; 0.5 mg/ml in borate buffer, pH 8.0; prepared as described by Banker and Cowan, 1977)–coated 35 mm tissue culture plates at 3.5×10^5/plate. After 2–4 hr the medium was replaced with a serum-free defined medium (N2; Bottenstein and Sato, 1979, as modified by Bartlett and Banker, 1984), and the coverslips containing the feeder glial cells were placed on top of each plate of pyramidal neurons. Cytosine-β-D-arabinofuranoside (5 μM) was added to each plate 2 d later to inhibit nonneuronal cell proliferation. For immunocytochemistry studies, the layers of glial and neuronal cells were inverted. Neurons were plated at 6×10^3 cells/cm on 15 mm round glass coverslips and allowed to adhere for 2–4 hr before being transferred to 60 mm dishes containing the supporting astrocytes attached to the bottom of the culture dish.

Polymerase chain reaction and Southern blot analysis. RNAs were isolated by the RNAzol method (Cinna/Biotecx) from cultured hippocampal pyramidal neurons and glial cells, PC12 cells, NIH3T3 cells, and NIH3T3-derived cell lines expressing $gp140^{trk}$, $gp145^{trkB}$, $gp95^{trkB}$, $gp145^{trkC}$ and $p75^{LNGFR}$ (Klein et al., 1991a,b; Lamballe et al., 1991; Jing et al., unpublished observations). PCR-aided amplification was performed on cDNAs derived from each of these RNAs by reverse transcription using avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim). Amplimers used in the PCR reactions were (1) *trk* ($gp140^{trk}$ transcripts): 5′ (5′-ATGTGGGGGACGACGTGCTG-3′) and 3′ (5′-GCAGACACCCGTGCCGCAC-3′) amplimers correspond to nucleotides 701–720 and 1105–1123, respectively, of the human *trk* cDNA clone (pLM6) described by Martin-Zanca et al. (1989); (2) *trk*B TK^+ ($gp145^{trkB}$ transcripts): 5′ (5′-TGAAGGACGCCAGCGACAATG-CACGCAAGG-3′) and 3′ (5′-GGTGAATTTCCTATACATGAT-GCTCTCTC-3′) amplimers correspond to nucleotides 2229–2258 and 2673–2701, respectively, of the mouse *trk*B TK^+ cDNA clone (pFRK43) described by Klein et al. (1989); (3) *trk*B TK^- ($gp95^{trkB}$ transcripts): 5′ (5′-GCTGGGGCTGTGGTGCTTGTTGCCTG-3′) and 3′ (5′-CT-TCGATTCTATATTTGAACTATTGTA-3′) amplimers correspond to nucleotides 1454–1479 and 1685–1711, respectively, of the mouse *trk*B TK^- cDNA clone (pFRK42) described by Klein et al. (1990); (4) *trk*C ($gp145^{trkC}$ transcripts): 5′ (5′-GGGTGTGTGGTGATGGTGAC-CCACTCATCATGGTC-3′) and 3′ (5′-GCCAAGAATGTCCAGGT-AGATCGGGGTGGCCTTCC-3′) amplimers were derived from a mouse *trk*C cDNA clone (pFL16) (Lamballe et al., 1933) and correspond to nucleotides 1845–1879 and 2472–2505, respectively, of the porcine *trk*C cDNA clone (pFL19) described by Lamballe et al. (1991); (5) LNGFR ($p75^{LNGFR}$ transcripts): 5′ (5′-GAGGGCACATACTCA-GACGAAGCC-3′) and 3′ (5′-GTCTATATGTTCAGGCTGGTA-ACC-3′) amplimers correspond to nucleotides 567–590 and 1206–1229, respectively, of the rat LNGFR cDNA clone (pNGFR.1) described by Radeke et al. (1987). PCR reactions were carried out for 40 cycles at an annealing temperature of 55°C using a Perkin Elmer-Cetus DNA thermal cycler as recommended by the manufacturer. The resulting PCR products were run on 2% NuSieve agarose gels (FMC) and transferred onto Genescreen Plus membranes (Du Pont–New England Nuclear) for Southern blot analysis. In all cases, the size of the amplified cDNA fragment corresponded to that predicted from the cDNA sequence and the set of amplimers used. Probes were generated by PCR-aided amplification of sequences internal to those cDNA sequences described above and labeled using the mutiprime DNA labeling system (Amersham). These were (1) *trk* ($gp145^{trk}$ transcripts): nucleotides 702–1092 of the rat *trk* cDNA sequence described by Meakin et al. (1992); (2) *trk*B TK^+ ($gp145^{trkB}$ transcripts): nucleotides 2270–2672 of the mouse *trk*B TK^+ cDNA clone (pFRK43) described by Klein et al. (1989); (3) *trk*B TK^- ($gp95^{trkB}$ transcripts): nucleotides 1480–1683 of the mouse *trk*B TK^- cDNA clone (pFRK42) described by Klein et al. (1990); (4) *trk*C ($gp145^{trkC}$ transcripts): nucleotides 1924–2241 of the porcine *trk*C cDNA clone (pF19) described by Lamballe et al. (1991); (5) LNGFR ($p75^{LNGFR}$ transcripts): nucleotides 594–1205 of the rat LNGFR cDNA clone (pNGFR.1) described by Radeke et al. (1987). Membranes were prehybridized for 30 min at 60°C in a solution consisting of 1% BSA, 500 mM $NaPO_4$, (pH 7), 7% SDS, 15% formamide, and 1 mM EDTA, hybridized for 6 hr at 60°C in the same solution supplemented with 100 mg/ml of single-stranded DNA, and washed twice for 30 min with 150 mM $NaPO_4$, (pH 7), 0.1% SDS at 60°C and once with 30 mM $NaPO_4$ (pH 7), 0.1% SDS.

In situ *hybridization.* A *trk*B-specific cRNA probe was synthesized as described by Klein et al. (1989) from a 483 base pair *Hinc*II DNA fragment of pFRK1 that encompasses sequences encoding a portion of the putative extracellular domain of $gp145^{trkB}$. This *Hinc*II DNA fragment was subcloned into the pGEM-3Z vector (Promega) to generate pFRK16. In order to synthesize a ^{35}S-labeled single-stranded antisense cRNA probe, pFRK 16 was linearized by digestion with *Hind*III and *in vitro* transcribed with T7 RNA polymerase (Promega) in the presence of ^{35}S-labeled UTP (>1000 Ci/mmol; Amersham). The resulting antisense probe was mildly degraded by alkaline hydrolysis to an average of 100–200 bases in length to facilitate its accessibility to the cells. *In situ* hybridization analysis was performed on the cultured pyramidal neurons using methods described by Kleiman et al. (1990).

Immunocytochemistry. Cultured pyramidal cells were fixed in HBSS containing 3% paraformaldehyde and 0.01% glutaraldehyde. Cells were rinsed three times in PBS, permeabilized in 0.1% Triton X-100/PBS solution, rinsed again, and blocked in 2% BSA in PBS for 45 min. Primary antibodies were diluted as indicated below and applied for

2–4 hr at room temperature. Immunocytochemical staining with rabbit polyclonal anti-TrkB (1:1,000; Klein et al., 1991b) or c-Fos (1:500; gift of T. Curran, Roche Institute of Molecular Biology) as well as anti-p75^{LNGFR} (192 monoclonal; 1:500; see Misko et al., 1987) was performed using commercial avidin–biotin peroxidase complex kits (Vector). The secondary antibody (biotin conjugated anti-rabbit or mouse) was diluted 1:1,000 and applied for 45 min at room temperature after washing. Avidin-biotinylated peroxidase reagent was mixed according to kit instructions and applied for 1 hr at room temperature, following which the cells were rinsed in PBS and then developed with brief exposure to diaminobenzidine–hydrogen peroxide.

PC12 cells, cultured as previously described (Nairn et al., 1987), were grown on coverslips, fixed, and stained as described above.

Immunoblotting. Pyramidal neuron proteins were extracted using sample buffer (0.5% SDS, 8 M urea, 50 mM 2-mercaptoethanol) and then separated by SDS–7.5% PAGE and electrophoretically transferred to nitrocellulose. The nitrocellulose was fixed in 10% isopropanol, 7% acetic acid, incubated with "Blotto" (5% w/v nonfat dried milk in PBS) for 1 hr, and incubated with anti-gp145^{trkB} antibody diluted 1:1000 or, when gp145^{trkB} immunoprecipitates were analyzed, anti-phosphotyrosine antibody (Upstate Biotechnology, Inc., Lake Placid, NY) diluted 1:2000 in PBS. The nitrocellulose was incubated with antibodies for 2 hr at room temperature. After washing in Blotto, the sheets were incubated in ^{125}I-protein A (1 μCi/ml). After further washes, immunoblots were exposed to x-ray film (Kodak XAR-5) with an intensifying screen and developed.

Mitogen-activated protein kinase activity assay. The glial layer was removed from the pyramidal neurons for 4 hr prior to experimentation. After glia deprivation, the pyramidal neurons were treated with recombinant BDNF (20 ng/ml; prepared as described by Cordon-Cardo et al., 1991) for the specified times, following which the cells were rinsed twice with HBSS and harvested in homogenization buffer (25 mM Tris, 1 mM EDTA, 0.5 mM EGTA, 5 mM 2-mercaptoethanol, 10 mM β-glycerophosphate, 2 mM pyrophosphate) to which 0.1 mM vanadate, 0.1 mM phenylmethylsulfonyl fluoride, and 0.02 mM leupeptin were added immediately before use. Samples were briefly sonicated, and the homogenate was subjected to ultracentrifugation at 100,000 × *g* for 30 min. Supernatants were collected and microtubule-associated protein (MAP) kinase activity was determined in triplicate by incubation of 50 μl of extract with 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM EGTA, 0.12 mg/ml microtubule-associated protein-2 (MAP2) or 0.25 mg/ml myelin basic protein (MBP), and 20 μM γ-^{32}P-ATP (1000 cpm/pmol; Amersham) in a final volume of 100 μl for 10 min at 30°C. MAP2 and MBP were isolated from bovine brain by established procedures. The reactions were stopped by the addition of 50 μl of SDS sample buffer. Samples were boiled for 1 min and subject to SDS-PAGE (6% for MAP2, 12.5% for MBP). After staining and destaining, bands corresponding to MAP2 or MBP were cut from gel and the radioactivity determined by liquid scintillation counting. Protein concentrations for each sample were determined using the dye-binding method (Bio-Rad), and used to calculate specific activities.

MAP kinase immunoprecipitation. Three 35 mm dishes, each containing approximately 3 × 10^5 cells in the neuronal plane (~8 μg protein/plate), were used per sample. Coverslips containing astrocytes were removed, and the neuronal layer was washed and equilibrated in low-phosphate HEPES-buffered saline (135 mM NaCl, 5 mM KCl, 1.26 mM CaCl, 10 mM glucose, 10 mM HEPES pH 7.4) at 30°C in a gently shaking water bath. $^{32}P_i$ (ICN Radiochemicals) was added to a final specific activity of 750 μCi/ml, and the incubation continued for 1 hr; BDNF was then added to some samples at a concentration of 20 ng/ml for 7 min. Each dish was removed from the shaker, the medium quickly aspirated, and after washes in ice-cold saline the cells were solubilized in 50 μl/plate SDS sample buffer (150 μl total volume/sample); 120 μl of the sample was added to NET buffer (150 mM NaCl, 50 mM Tris, and 1 mM EDTA) containing 5% NP40 and 0.2% BSA (440 μl final volume). Samples were preincubated for 30 min on ice with 50 μl of a 10% suspension of washed protein A–bearing *Staphylococcus aureus* cells (IgGsorb, Enzyme Center); 5 μl of anti-MAP kinase antiserum (polyclonal, prepared against amino acids 309–326 of the predicted ERK-1 sequence) was added to the precleared supernatants and allowed to react for 2 hr on ice; 50 μl of IgGsorb (10%) was then added, and incubation continued for 30 min on ice. IgGsorb was pelleted and washed three times with NET buffer containing 0.1% NP40. The final pellet was then solubilized in 120 μl of SDS sample buffer, boiled for 1 min, and resolved on 10% gels by SDS-PAGE.

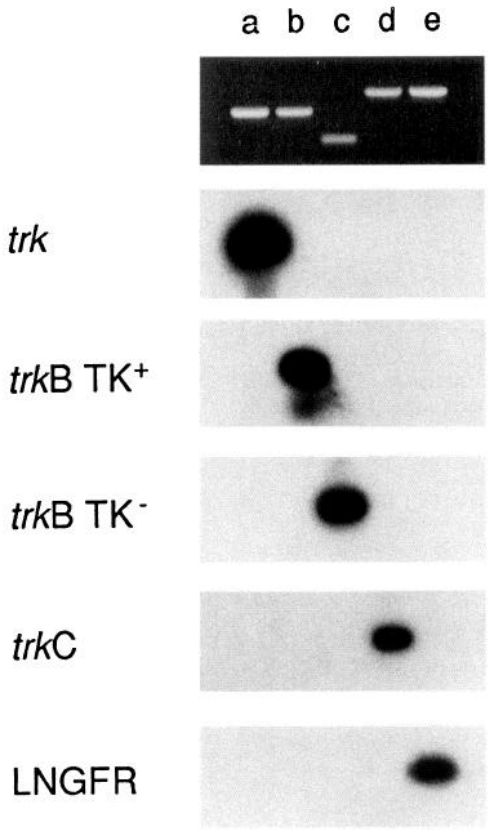

Figure 1. Specificity of the primers used to amplify *trk* gene sequences in PCR analysis. cDNA prepared from total mouse brain mRNA was incubated with amplimers corresponding to the cytoplasmic sequences of *trk* (*lane a*), *trk*B TK$^+$ (*lane b*), *trk*B TK$^-$ (*lane c*), *trk*C (*lane d*), and LNGFR (*lane e*), and the amplified DNA sequences were separated by gel electrophoresis (*top*). These PCR-amplified DNA fragments were submitted to Southern blot analysis using as probes specific cDNA sequences derived from pLM6 (*trk*), pFRK43 (*trk*B TK$^+$), pFRK42 (*trk*B TK$^-$), pFL19 (*trk*C), and pLL4 4 (LNGFR) (see Materials and Methods).

Results

Expression of neurotrophin receptors in hippocampal pyramidal cultures

To establish whether the receptors for the NGF family of neurotrophins are expressed in cultures of hippocampal pyramidal neurons, we utilized PCR-aided amplification of sequences corresponding to the known members of the *trk* gene family of tyrosine protein kinases including the Trk receptor gp140^{trk}, the TrkB receptors gp145^{trkB} and its noncatalytic isoform gp95^{trkB}, and the TrkC receptor gp145^{trkC}, as well as to the low-affinity receptor p75^{LNGFR}. As illustrated in Figure 1, each of the primers used in this study specifically amplified those sequences corresponding to the neurotrophin receptor from which they were derived. In addition, these primers efficiently amplified cDNAs generated by reverse transcription of RNAs isolated from NIH3T3 cells ectopically expressing each of these receptors, but not from the parental NIH3T3 cells (Fig. 2, lanes b, c). As an additional specificity control, RNA obtained from rat PC12 pheochromocytoma cells only yielded detectable amplification in those samples incubated with amplimers for *trk* and LNGFR (Fig. 2, lane f), the only neurotrophin receptors known to be expressed in these cells.

We next utilized this PCR-based strategy to determine which

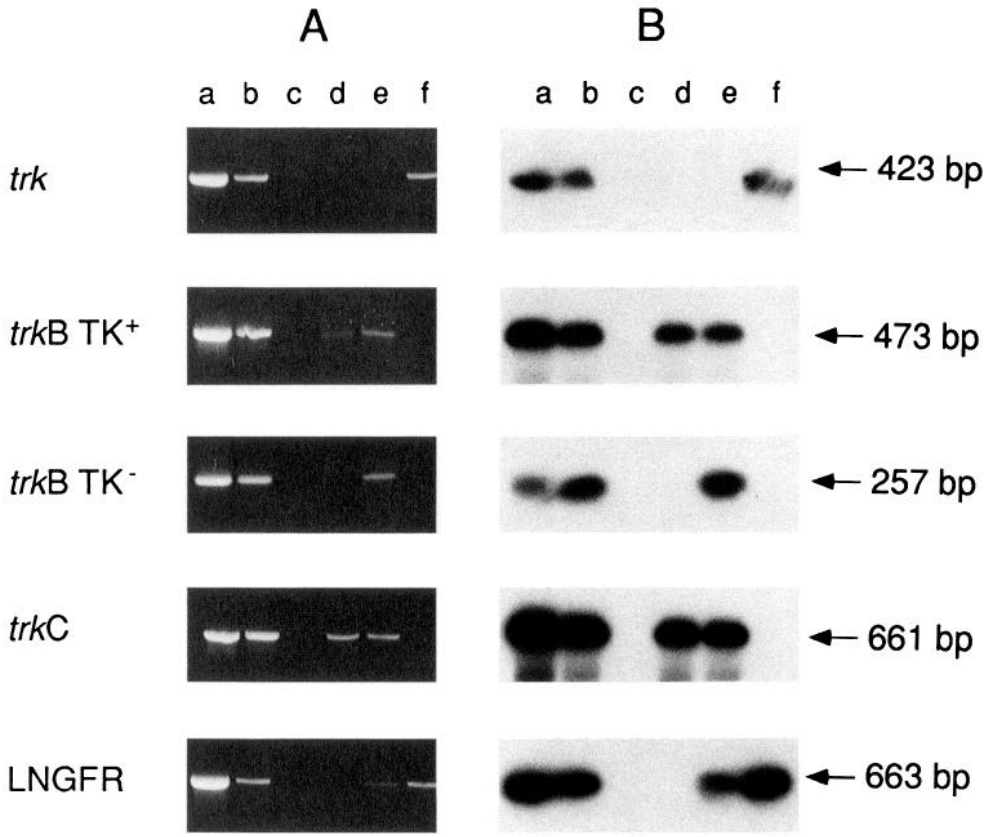

Figure 2. Expression of the LNGFR and each of the known members of the *trk* family of tyrosine kinase receptors in hippocampal pyramidal cell cultures. *A*, Ethidium bromide staining of gel-electrophoresed PCR-amplified DNA from plasmids encoding specific cDNA clones for the indicated receptors (*lane a*) (see Fig. 1 caption), and cDNAs prepared from RNAs isolated from NIH3T3 cells expressing the receptors encoded by each of the indicated genes (*lane b*), parental NIH3T3 cells (*lane c*), hippocampal pyramidal neurons (*lane d*), supportive glial cell cultures derived from the cerebral hemispheres (*lane e*), and PC12 cells (*lane f*). *B*, Southern blot analysis of the DNA samples shown in *A* using as probes those described for Figure 1.

neurotrophin receptors are expressed in the hippocampal pyramidal neurons. RNA was isolated from cells after 2 d in culture, when more than 90% of the cells in the neuronal plane exhibited neurites. RNA was also prepared from cultured astroglia, the cells that are derived from the cerebral hemispheres of postnatal rats. In each case, the size of the amplified DNAs corresponded to that expected from the known cDNA sequence and the set of amplimers used (Fig. 2*A*). Moreover, these amplified cDNAs hybridized very efficiently with specific internal probes derived from the corresponding cDNA clones, indicating the correct origin of the amplified sequences (Fig. 2*B*). As shown in Figure 2, lane d, the pyramidal neurons yielded amplification in those samples incubated with *trk*B TK$^+$- and *trk*C-derived primers but not those containing *trk*, *trk*B TK$^-$, or LNGFR amplimers. Similar results were obtained with the RNA isolated from glial cultures. DNA amplification could be observed in those samples incubated with *trk*B and *trk*C amplimers, but not in those containing *trk* primers. On the other hand, glial cell cultures contained detectable levels of LNGFR as well as *trk*B TK$^+$ and *trk*B TK$^-$.

In an effort to determine whether there might be low levels of p75^{LNGFR} amplified DNA in those samples derived from the neuronal layer, we performed a double PCR analysis using internal primers. No DNA could be detected even after a second-tier amplification of 50 cycles (data not shown). These results strongly suggest that the LNGFR gene is not expressed in the embryonic hippocampal pyramidal neurons used here. Finally, amplified DNA corresponding to *trk*B TK$^-$ was also undetectable in the neuronal layer (Fig. 2*B*, lane d). These results suggest that this noncatalytic TrkB receptor may be specifically expressed in glial cells.

Further confirmation of the presence of *trk*B transcripts in pyramidal neurons was obtained by *in situ* hybridization. ^{35}S-labeled antisense and sense cRNA probes corresponding to nucleotides 1181–1663 in the *trk*B cDNA clone pFRK43 (Klein et al., 1989) were prepared as described in Materials and Methods. These probes were selected as they do not cross-react, even under low-stringency conditions, with *trk* or *trk*C sequences (Klein et al., 1989; Lamballe et al., 1991) and are not present in the gp95^{trkB}-encoding transcripts. Anti-sense labeling of pyramidal neurons (Fig. 3*A,B*) showed silver grain clusters over the cell bodies of most neurons. No clustering of silver grains was found on cells incubated with the sense probe (Fig. 3*D*).

Immunological detection of the gp145^{trkB} but not of p75^{LNGFR} receptors in hippocampal pyramidal cultures

Immunocytochemical analysis of 10-d-old pyramidal cell cultures was carried out with affinity-purified anti-gp145^{trkB} antibodies derived from a rabbit polyclonal antiserum made against a peptide corresponding to amino acid residues 794–808 of mouse gp145^{trkB} (Klein et al., 1990). This antiserum has been previously shown to recognize gp145^{trkB} in fibroblasts transfected with *trk*B and does not cross-react with either gp140^{trk}, gp145^{trkC}, or gp95^{trkB} (data not shown). More than 90% of cells stained for gp145^{trkB} in the hippocampal pyramidal neurons and immunostaining was visualized in both the cell body and processes (Fig. 4).

In parallel, we conducted immunolabeling for p75^{LNGFR} with the well-characterized mouse monoclonal antibody 192 (Chandler et al., 1984); PC12 cells were used as a positive control and compared with pyramidal neurons maintained in culture for 10

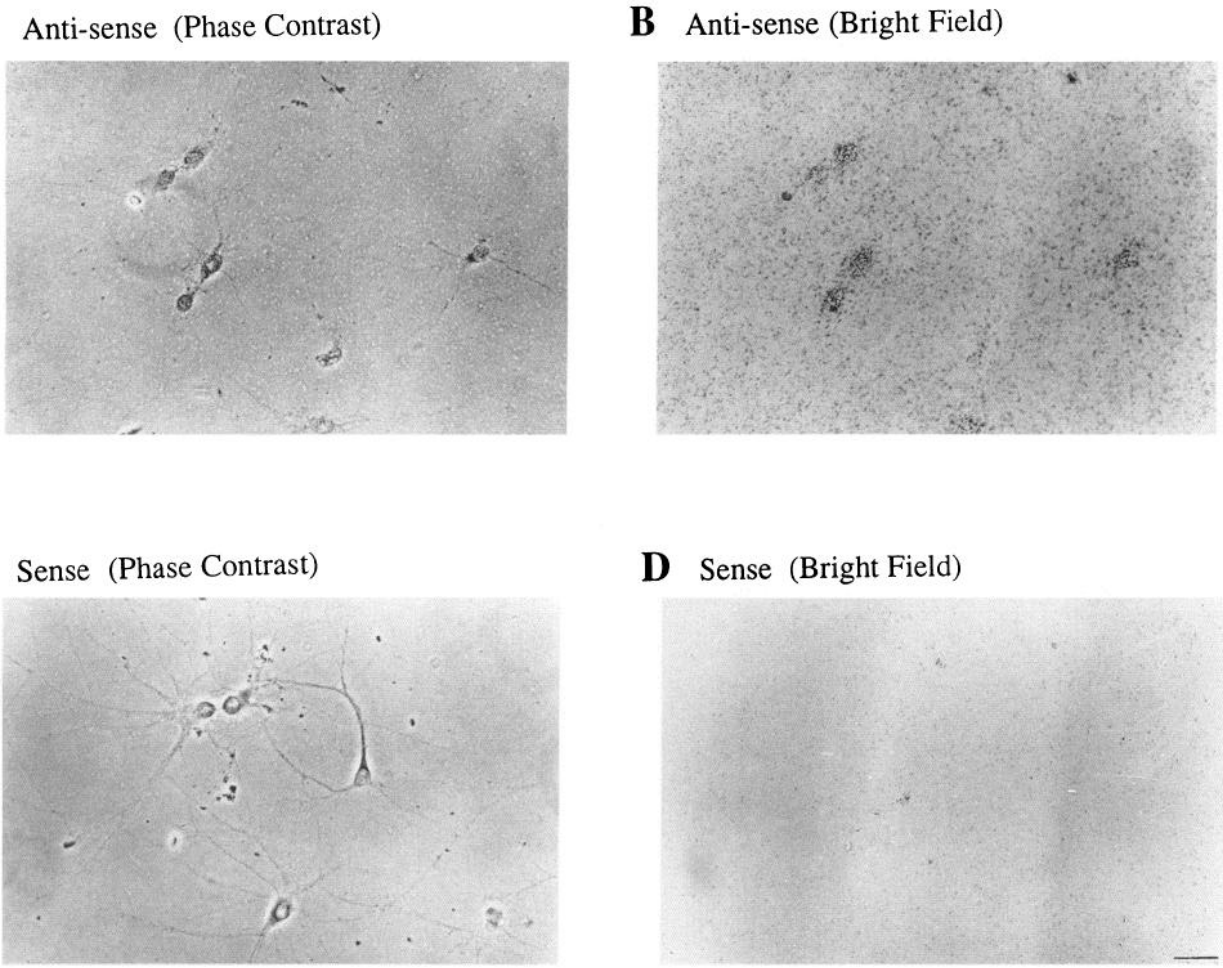

Figure 3. *In situ* hybridization showing *trk*B mRNA in cultured hippocampal pyramidal neurons. *A* and *B*, Anti-sense labeling of pyramidal neurons. *A* shows a phase-contrast image; *B* shows the same field using bright-field illumination to show silver grains. Clustering of silver grains can be detected in the cell bodies of most neurons. *C* and *D*, Sense labeling of pyramidal neurons: corresponding phase-contrast and bright-field illumination. The procedure for mRNA detection was as described by Kleiman et al. (1990). Scale bar, 20 μm.

d. As depicted in Figure 5*A*, PC12 cells stained intensely and uniformly for $p75^{LNGFR}$. In contrast, no demonstrable staining for this antigen was found in pyramidal neurons (Fig. 5*B*). The lack of detectable protein in the pyramidal neurons, along with the PCR amplification studies described above, support our contention that this low-affinity neurotrophin receptor is completely absent in the neuronal plane of these cultures.

To verify the immunocytochemical staining of $gp145^{trkB}$ and to determine the molecular weight of the recognized protein, immunoblot analysis was performed with the same antibody. As shown in Figure 6, an immunoreactive protein of ~140 kDa was present in samples derived from both total adult hippocampus and pyramidal neurons in culture for 10 d, but was not detected in cultured astroglia.

BDNF stimulates tyrosine phosphorylation of $gp145^{trkB}$ receptors

Recent studies have shown that BDNF and NT-3 both induce the rapid tyrosine phosphorylation of $gp145^{trkB}$ in fibroblasts transfected with *trk*B (Klein et al., 1991b; Squinto et al., 1991). To examine whether BDNF could induce a similar response in hippocampal pyramidal cells, we incubated 10-d-old neuronal cultures (glial deprived for 4 hr) with BDNF (20 ng/ml) for 5 min and assessed changes in the level of phosphotyrosine in immunoprecipitates of $gp145^{trkB}$ with anti-phosphotyrosine antibodies. As shown in Figure 7, BDNF stimulates the phosphorylation of tyrosine residues in $gp145^{trkB}$.

BDNF-induced signal transduction in pyramidal neurons

Two bioassays were examined to determine whether BDNF initiates a cascade of signal transduction: the activation of MAP kinases, which is a relatively rapid effect (measured in minutes), and the increased expression of c-*fos*, a longer-term effect (measured in hours). Figure 8 shows the time course of BDNF effects on MAP kinase activation *in vitro*. In this experiment MAP kinase activity was determined using MAP2 as substrate; in other experiments the use of MBP as substrate gave similar results (data not shown). In preliminary experiments we found it necessary to remove the pyramidal neurons from glia 4 hr prior to experimentation in order to lower basal levels of kinase activity. Activation of MAP kinase was shown to peak 2 min after BDNF addition, at 300% of the control level, and to return to 150% control level by 45 min. When MAP kinases were immunoprecipitated from ^{32}P-prelabeled cultures using a polyclonal antibody raised against amino acids 309–326 in the ERK1 sequence (Boulton et al., 1990), an increase in the phosphory-

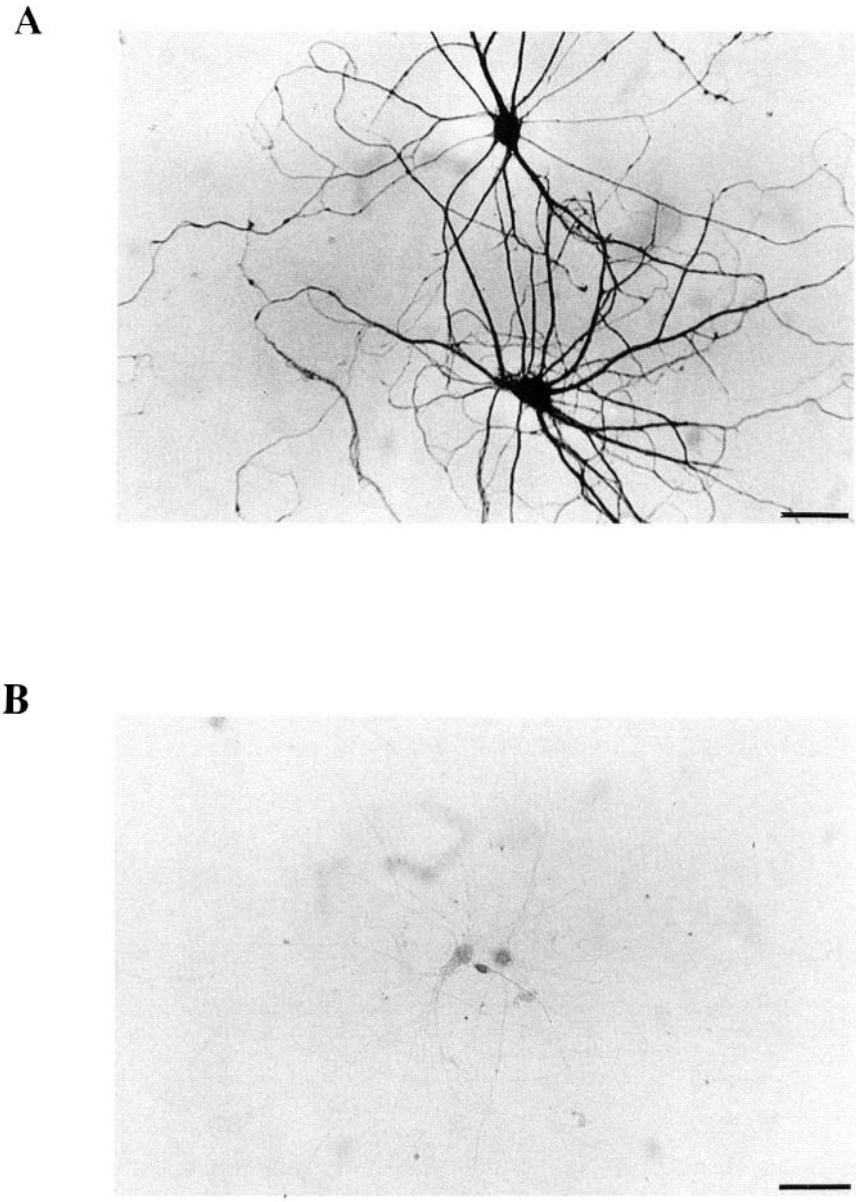

Figure 4. Immunocytochemistry of gp145^{trkB} in cultured pyramidal neurons. *A*, Neurons, in culture for 10 d, were reacted with an affinity-purified anti-gp145^{trkB} rabbit polyclonal antibody. The immunological reaction was visualized using biotinylated secondary antibody followed by an avidin–biotinylated HRP complex with diaminobenzidine tetrachloride and hydrogen peroxide as substrate. *B*, Control in which normal rabbit IgG was used as the primary antibody. Scale bars, 20 μm.

lation of two major species of 41 and 44 kDa was shown to occur (Fig. 8, inset).

BDNF was also found to induce the expression of c-*fos* in a majority of the pyramidal neurons (Fig. 9). Pyramidal neurons were glia deprived for 4 hr, and then treated with BDNF (20 ng/ml) for various times and expression of nuclear c-*fos* visualized by immunocytochemistry. Untreated cultures exhibited virtually no c-Fos–positive neurons (Fig. 9*A*). BDNF-induced c-*fos* expression peaked at 30 min (Fig. 9*B*), continued to be elevated at 60 min (Fig. 9*C*), and declined to near control levels at 120 min (Fig. 9*D*). Cell counts revealed that >90% of the cells responded at 30 and 60 min.

Discussion

The recent emergence of the Trk family of receptor tyrosine kinases (RTKs) as the probable functional receptors for the NGF family of neurotrophins emphasizes the importance of tyrosine phosphorylation in the CNS. Recent results employing degenerate probes suggest widespread distribution of several types of RTKs in the rat brain (Lai and Lemke, 1991). Additionally, certain nonreceptor tyrosine kinases such as c-Src are abundant in central neurons (Sugrue et al., 1990). The existence of such enzymes in postmitotic neuronal populations suggests that tyrosine phosphorylation plays an important role in CNS functions other than cell proliferation. Thus, it is clearly relevant to study the downstream events initiated by RTK activation in neuronal tissue. However, most studies on neurotrophin signal transduction have been performed on proliferating cell lines or transfected non-neuronal cells. In this study we have focused on the BDNF–TrkB system in cultured hippocampal pyramidal neurons. These cultures contain a relatively homogeneous population of primary neurons and have the advantage that biochemical experiments are readily performed after separation of the neuronal and glial layers (Scholz and Palfrey, 1991).

Previous studies indicated that *trk*B mRNA is present in the pyramidal cell layer of the adult mouse hippocampus (Klein et al., 1989). In the present study, we extended these findings by demonstrating that *trk*B transcripts as well as the tyrosine kinase receptor gp145^{trkB} are present in cultured rat embryonic hip-

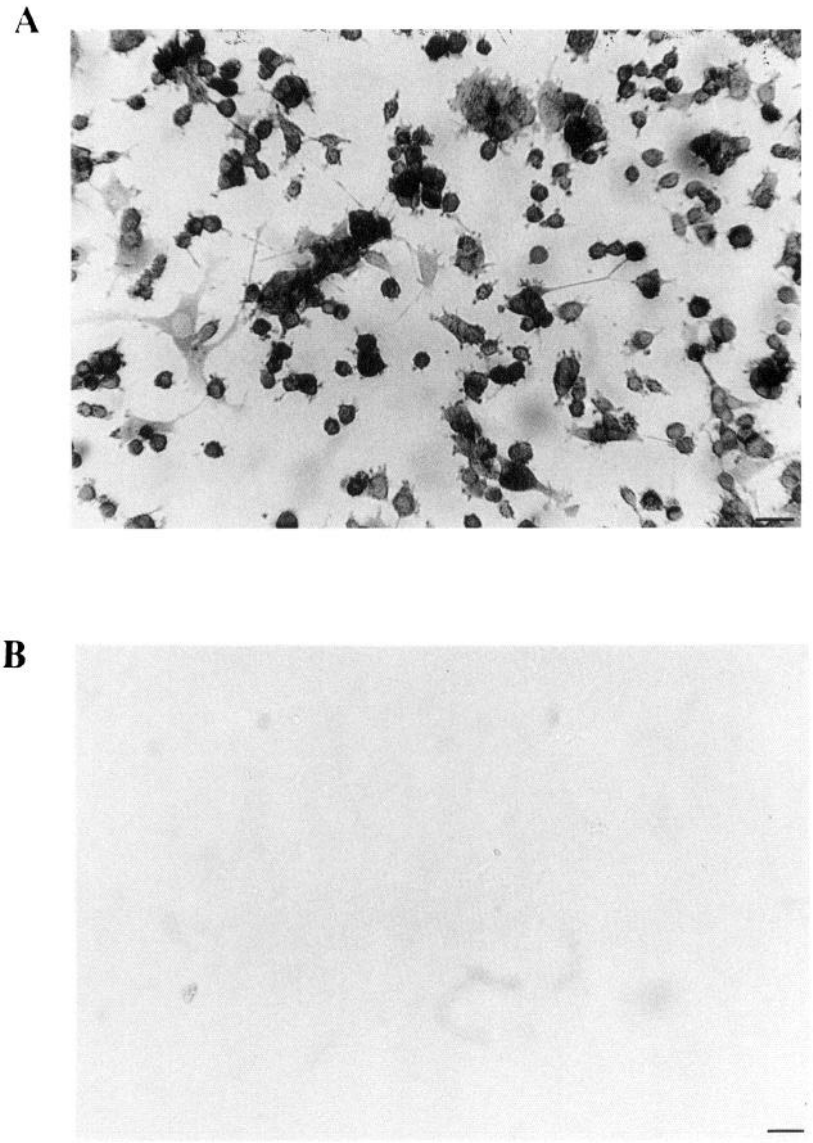

Figure 5. Immunocytochemical localization of gp75^{LNGFR} in PC12 cells and pyramidal neurons. PC12 cells (*A*) and pyramidal cells (*B*) were reacted with a mouse monoclonal antibody (clone 192) against gp75^{LNGFR}. The reaction was visualized as described for Figure 4. Scale bars, 20 μm.

pocampal pyramidal neurons. *In situ* hybridization studies revealed that *trk*B mRNA could be visualized in the cell bodies of most pyramidal neurons. Over 90% of the cells stained intensely for gp145^{trkB} using affinity-purified antibodies, and the antigen was found to be widely distributed in both the processes and cell bodies of these neurons. Immunoblot analysis revealed a single cross-reacting protein of ~140 kDa. This molecular weight agrees well with that found for the receptor in *trk*B-transfected fibroblasts (Klein et al., 1989, 1991b). Our PCR studies strongly support the concept that this population of neurons do not express either gp140^{trk} or the low-affinity receptor p75^{LNGFR} (see below). The absence of p75^{LNGFR} expression was supported by immunocytochemical analysis of the neuronal layer. These data confirm that the BDNF receptor, gp145^{trkB}, is present in the pyramidal cell layer of the hippocampus at an early stage of development. It is interesting to note that the hippocampus also expresses high levels of BDNF, suggesting that a local trophic factor network may exist. Whether BDNF and its receptor are present in the same cells, providing a potential autocrine loop (cf. Ernfors et al., 1990), is yet to be determined.

One of the hallmarks of RTKs is their autophosphorylation on certain tyrosine residues upon binding of their cognate ligand (Ullrich and Schlessinger, 1990). For example, NGF, BDNF, and NT-3 have all been shown to induce tyrosine phosphorylation of their cognate receptors gp140^{trk}, gp145^{trkB}, and gp145^{trkC} in transfected PC12 and heterologous cells (see Barbacid, 1993, for a review). In the present study, BDNF was shown to induce tyrosine phosphorylation of gp145^{trkB} within 5 min, suggesting that gp145^{trkB} is the functional receptor that mediates BDNF-induced signal transduction.

Many downstream effects of NGF, the first neurotrophin to be discovered, have been well characterized in PC12 cells. Some early NGF-induced effects include a rapid increase in tyrosine kinase activity (Maher, 1988), activation of several enzymes including MAP kinases (e.g., Boulton et al., 1991) and the induction of c-*fos* and other early growth response genes (for review, see Morgan and Curran, 1991). A major issue is whether signal transduction events that transpire in neurotrophin-sensitive, differentiated postmitotic neurons are similar to those that mediate, for example, the differentiating effect of NGF in PC12 cells. As a first step in such an analysis, we examined some early signal transduction events initiated by BDNF in pyramidal neurons.

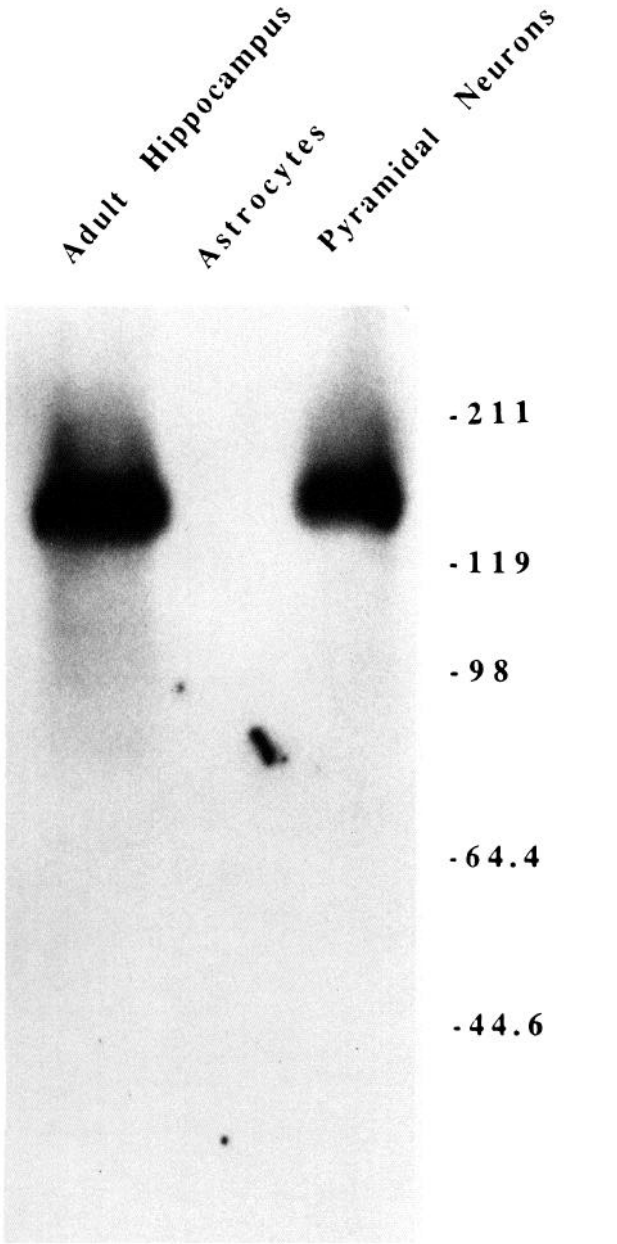

Figure 6. Western blot analysis of gp145^{trkB} in adult hippocampus, supportive astrocyte layer, and pyramidal neurons. Total hippocampus (100 μg, *lane 1*), astrocyte (80 μg, *lane 2*), and 10 d pyramidal neuron (80 μg, *lane 3*) proteins were separated by SDS-PAGE, transferred to nitrocellulose, and incubated in anti-gp145^{trkB} antibody. The immunological reaction was visualized utilizing ^{125}I-protein A followed by autoradiography.

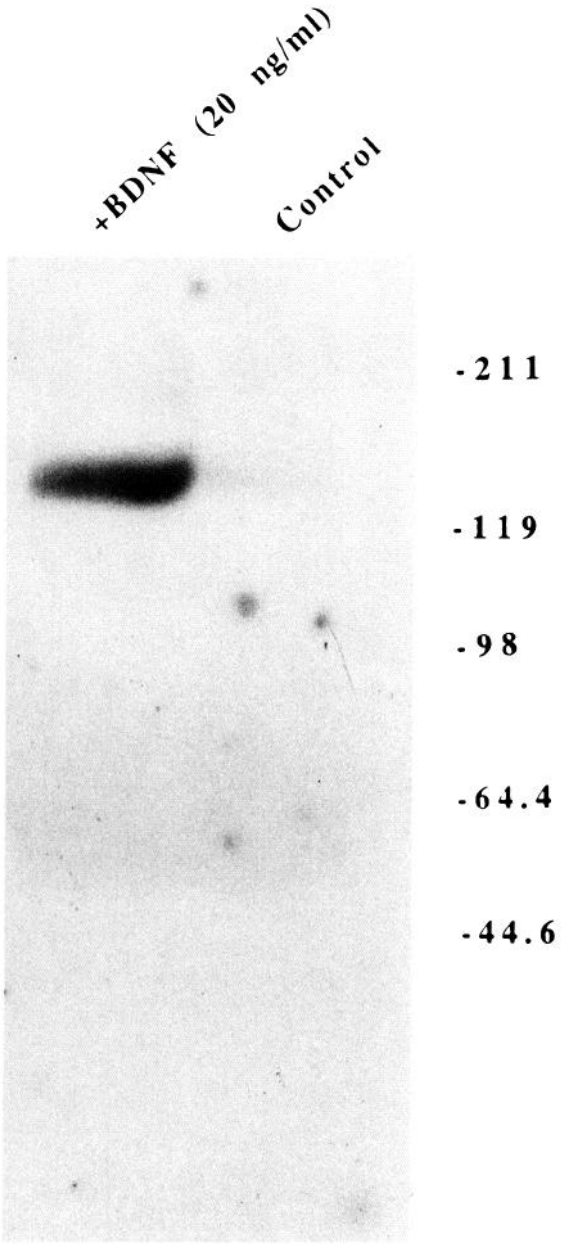

Figure 7. Immunoblot analysis of phosphotyrosine residues in gp145^{trkB} immunoprecipitates. Pyramidal neurons in culture for 14 d either were left untreated (*lane 2*) or were treated for 5 min with 20 ng/ml BDNF (*lane 1*). Anti-gp145^{trkB} antibodies were utilized to immunoprecipitate gp145^{trkB}. Immunoprecipitates, separated by SDS-PAGE, were transferred to nitrocellulose and incubated in monoclonal anti-phosphotyrosine antibodies (Upstate Biotechnology Inc.). Visualization of immunological reaction was by incubation with ^{125}I-protein A followed by autoradiography.

some early signal transduction events initiated by BDNF in pyramidal neurons.

BDNF rapidly stimulates MAP kinases in cultured hippocampal pyramidal cells. MAP kinases are serine/threonine kinases that are activated in a variety of cells in response to growth factors (see Sturgill and Wu, 1991, for review). These enzymes can use MAP2 or MBP as substrates, and phosphorylate a number of other important regulatory proteins including the transcription factor c-*jun* (Pulverer et al., 1991), the catecholamine biosynthetic enzyme tyrosine hydroxylase (Haycock et al., 1992), as well as other protein kinases such as S6 kinase II (p90^{rsk}; Chung et al., 1991). Two very similar forms of MAP kinase of 41 kDa and 44 kDa have been purified from both fibroblasts and PC12 cells (Boulton and Cobb, 1991; Sturgill and Wu, 1991). Activation of both forms of MAP kinase requires both threonine and tyrosine phosphorylation (Anderson et al., 1990; Boulton and Cobb, 1991). Both the 41 kDa and 44 kDa forms have been cloned from a rat brain cDNA library and termed ERK-2 and ERK-1, respectively (Boulton et al., 1990). The importance of such enzymes in the CNS is predicted by the

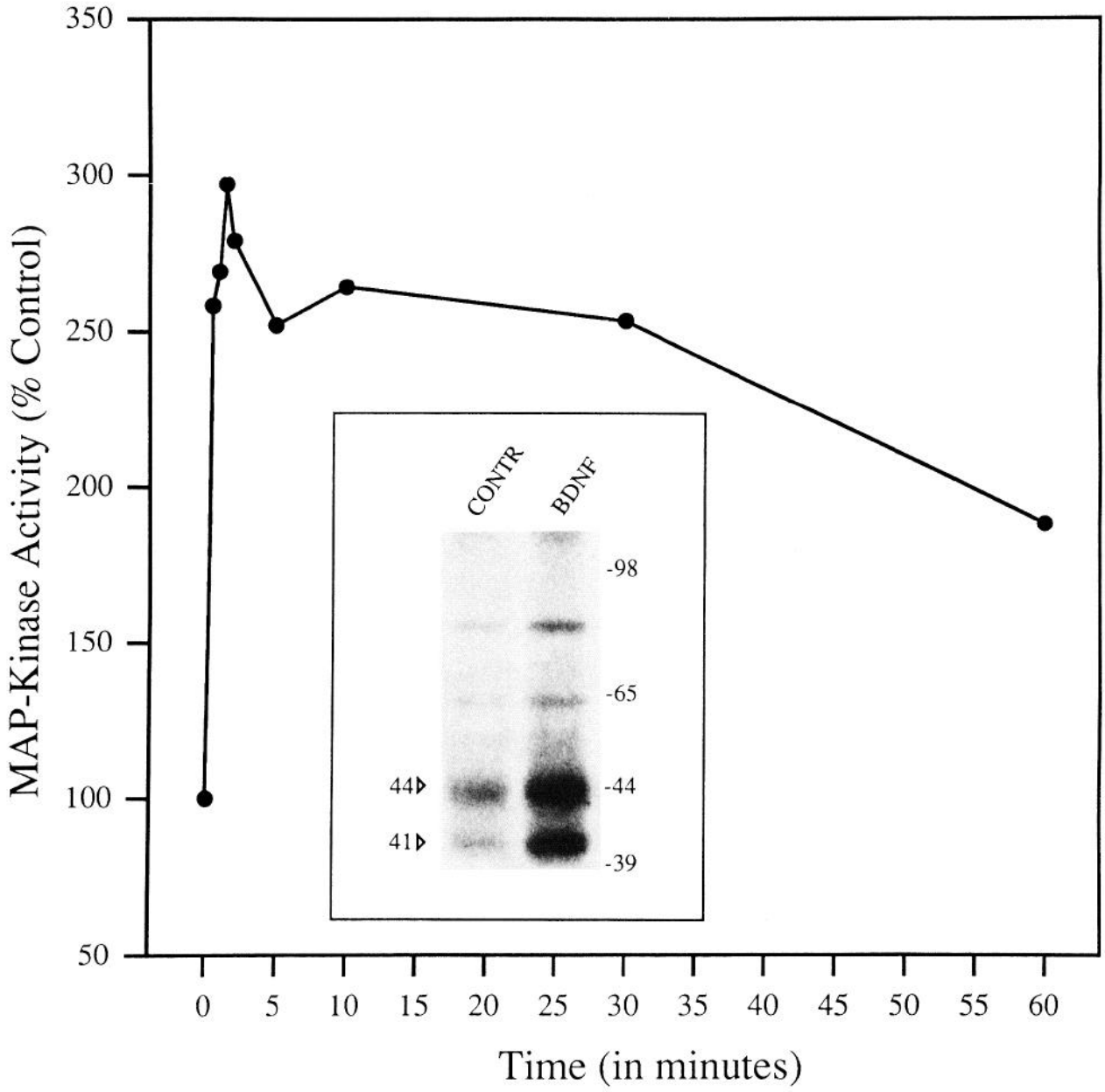

Figure 8. Activation of MAP kinases by BDNF. The *graph* depicts the time course of BDNF effects on the ability of cytoplasmic extracts to phosphorylate MAP2. Pyramidal neurons were glia deprived 4 hr prior to experimentation. Cells were treated with BDNF (20 ng/ml) for the indicated times and harvested, and then cell-free cytosolic extracts were prepared and assayed as described in Materials and Methods. Phosphorylated MAP2 was isolated by SDS-PAGE, excised from gel, and scintillation counted. The *inset* demonstrates BDNF stimulated phosphorylation of MAP kinase isotypes immunoprecipitated from ^{32}P-prelabeled cultured pyramidal neurons. Extracts were prepared from control and BDNF (20 ng/ml, 7 min)–treated cultures and immunoprecipitated with a polyclonal anti-MAP kinase peptide antibody. BDNF stimulated a marked increase in the phosphorylation of proteins of 41 kDa and the 44 kDa, the presumptive ERK2 and ERK1 homologs in these cells. The identity of the minor higher-molecular-weight bands is unknown.

relative abundance of their mRNAs in the brain (Boulton et al., 1991b).

To assess the activation of MAP kinases in pyramidal neurons, two types of assays were employed: phosphorylation of specific substrate proteins MAP2 and MBP *in vitro* and immunoprecipitation of the phosphorylated isoforms of MAP kinases. The activation of MAP kinases was found to be rapid, reaching a maximum at 2 min and then returning toward baseline slowly over the next 60 min (Fig. 8). This time course resembles that found in a number of other systems (Sturgill and Wu, 1991). Immunoprecipitation revealed that the phosphorylation of both the 41 kDa and 44 kDa forms of the enzyme was stimulated by BDNF. It was found that the neuronal layer needed to be removed from the supportive astrocyte layer for several hours in order to lower basal levels of MAP kinase activity prior to the addition of BDNF. It appears, therefore, that the activity of MAP kinases in the neuronal plane may be maintained at an elevated level by factors secreted by astrocytes. The nature of these factors is not known, but they could conceivably include one or more members of the neurotrophin family shown to be synthesized by cultured astrocytes. As it has recently been shown that glutamate can upregulate BDNF and

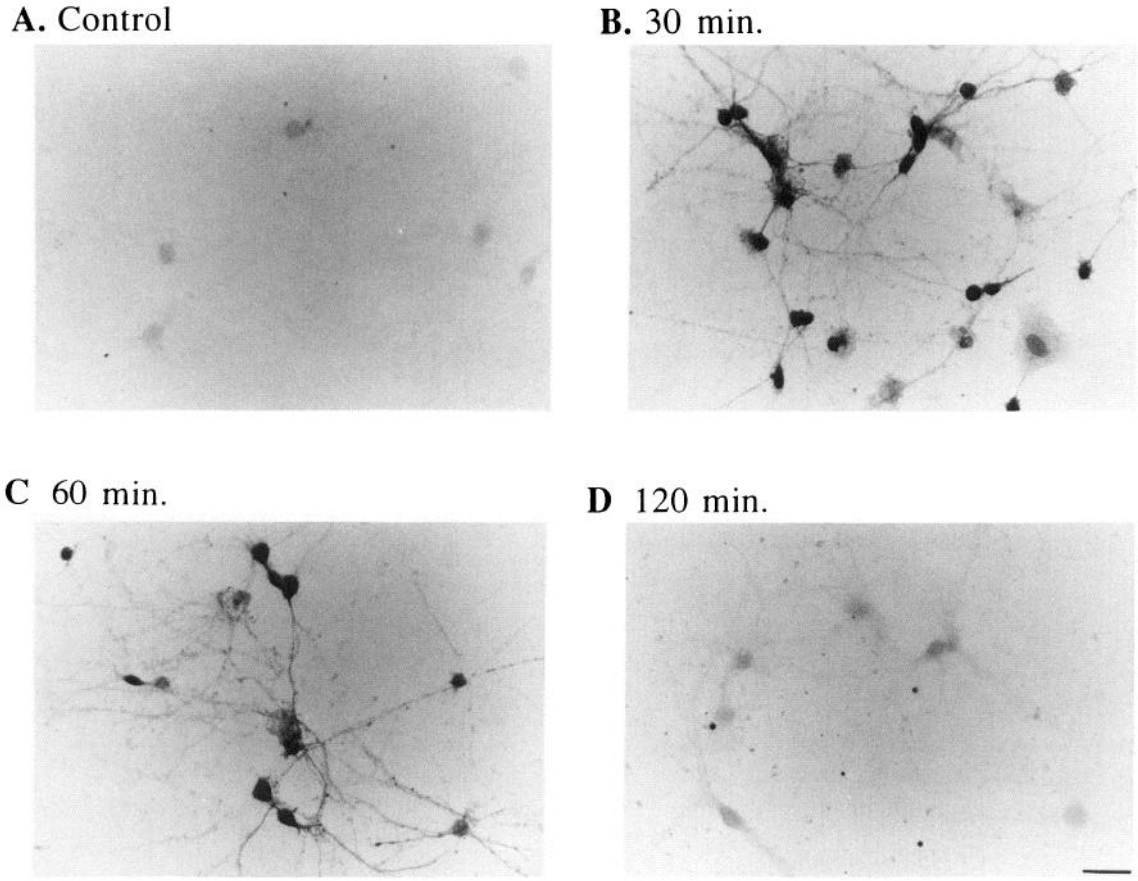

Figure 9. Immunocytochemistry of BDNF-induced expression of c-Fos. Pyramidal neurons, in culture for 10 d, were glia deprived for 4 hr and then treated with BDNF (20 ng/ml) for 0 min (*A*), 30 min (*B*), 60 min (*C*), and 120 min (*D*). The neurons were fixed and reacted with a rabbit polyclonal antibody directed against c-Fos. The immunological reaction was visualized using a biotinylated secondary antibody followed by an avidin–biotinylated HRP complex with diaminobenzidine tetrachloride as substrate. An increase in Fos was seen in >90% of the cells. Scale bar, 20μm.

NGF expression in cultured hippocampal neurons (Zafra et al., 1990) and can also stimulate MAP kinases in mixed hippocampal neuronal–glial cultures (Bading and Greenberg, 1991), these enzymes may provide a juncture for interaction of signals arising from neurotransmitters and neurotrophins in the nervous system.

To determine whether BDNF exerts longer-term effects on pyramidal neurons, induction of the early-response gene c-*fos* was examined. c-*fos* has been shown to be induced by a variety of stimuli in many types of cells, including neurons (for review, see Morgan and Curran, 1991). Previously, NGF has been found to induce c-*fos* expression in PC12 cells (Milbrandt, 1986), cultured primary rat embryonic septal neurons (Downen et al., 1993) and to exert a permissive effect on expression of the transcription factor in cultured rat sympathetic neurons (Buckmaster et al., 1991). BDNF has been shown to induce c-*fos* expression in fibroblasts transfected with *trk*B (Klein et al., 1991b) as well as in E18 embryonic hippocampal cultures (Collazo et al., 1992). In the pyramidal neurons, BDNF induction of c-Fos expression peaked at 30–60 min and returned to near basal levels at 2 hr. The long-term effect on Fos expression indicates that BDNF initiates a signal transduction cascade that extends over a period of hours. The relevance of c-*fos* expression in a variety of postmitotic cells responding to diverse stimuli is poorly understood. The protein Fos can combine with members of the Jun family of gene products to form transcriptional activators of the AP-1 type (Morgan and Curran, 1991). There is some evidence for differential activation of different members of the Jun family in response to distinct stimuli in the CNS (e.g., Mellström et al., 1991) and the composition of AP-1 appears to affect the specificity of gene transcription (Morgan and Curran, 1991). Clearly, a major task in unraveling the effects of trophic factors in the CNS will be to determine the specific pathways of gene expression that lead to the survival of target neuronal populations. Currently, we are examining whether BDNF can enhance the survival of the cultured pyramidal neurons, in the absence of the astroglia layer.

One of the major questions in the neurotrophin receptor field is the role of $p75^{LNGFR}$ (for reviews, see Bothwell, 1991; Thoenen, 1991; Mallett and Barclay, 1991). Certain experimental evidence suggests that $p75^{LNGFR}$ is an essential component of the functional HNGFRs. For instance, expression of the LNGFR gene in a PC12 cell mutant lacking NGF receptors resulted in the appearance of low- and high-affinity NGF binding sites and of NGF responsiveness (Hempstead et al., 1989, 1990). More recent studies have shown that high-affinity NGF binding requires coexpression of $p75^{LNGFR}$ and $gp140^{trk}$ receptors. Considering that $p75^{LNGFR}$ is a common receptor for all the members of the NGF neurotrophin family, these findings have led to the hypothesis that the high-affinity neurotrophin receptors may be heterodimers of $p75^{LNGFR}$ and the various Trk receptors. According to this notion, the Trk receptor kinases would convey

ligand specificity and mediate signal transduction but p75^{LNGFR} would be an essential component in producing the high-affinity binding sites known to be required for biological activity (Bothwell, 1991; Thoenen, 1991).

Recent evidence contradicts such a model. For instance, gp140^{trk} receptors can bind NGF with picomolar affinity in the absence of p75^{LNGFR} (Klein et al., 1991a). Moreover, coexpression of an equal number of p75^{LNGFR} and gp140^{trk} receptors has no effect on either NGF affinity or NGF-mediated signal transduction in 3T3 cells (Jing et al., 1992). However, it could be argued that these and related studies (Glass et al., 1991; Ip et al., 1993) may not be biologically representative of the situation in neurons since they have been conducted in non-neuronal cells. More recently, Ibanez et al. (1992) have reported that a mutant NGF that has lost its ability to bind to p75^{LNGFR}, but not to gp140^{trk}, retains biological activity. These findings, however, could still be compatible with the heterodimeric p75^{LNGFR}–gp140^{trk} model only if the assumption is made that p75^{LNGFR} does not contribute significantly to the formation of the NGF binding site. Finally, one more compelling piece of evidence suggesting that the Trk RTKs are themselves functional has come from recent studies indicating that depolarized MAH cells, an immortalized sympathoadrenal progenitor cell line, acquire NGF responsiveness upon expression of gp140^{trk} but not p75^{LNGFR} receptors (Birren et al., 1992).

In the present report, we have definitively shown that gp145^{trkB}, but not p75^{LNGFR} receptors, are expressed in primary cultures of hippocampal pyramidal neurons. The presence of gp145^{trkB} was convincingly demonstrated by four different methods: PCR analysis, *in situ* hybridization, immunocytochemical staining, and immunoblot analysis. Whereas it is considerably more difficult to demonstrate conclusively the complete absence of p75^{LNGFR}, our negative immunostaining data and, in particular, the lack of detection of LNGFR transcripts using the extremely sensitive double-tier PCR amplification technique strongly suggest that hippocampal pyramidal neurons do not express p75^{LNGFR}. If so, the BDNF-initiated signal transduction events observed in the pyramidal neurons must have been exclusively mediated solely by gp145^{trkB} receptors. Considering the limited levels of expression of p75^{LNGFR} in the brain, it seems likely that the Trk receptor family plays a predominant role in neurotrophin-mediated effects in the CNS. The lack of detectable change in CNS phenotypes of transgenic mice lacking the LNGFR gene supports this concept (Lee et al., 1992). However, definitive conclusions regarding the role of the Trk receptors in the nervous system must await the generation of null mice in any of the three known *trk* loci.

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