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Brain-Derived Neurotrophic Factor Effects on Oligodendrocyte Progenitors of the Basal Forebrain Are Mediated Through TrkB and the MAP Kinase Pathway

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Abstract

Previous work has indicated that BDNF increases the differentiation of basal forebrain (BF) oligodendrocytes (OLGs) in culture through the mediation of trkB and the MAPK pathway (Du et al. [2006a,b] *Mol. Cell. Neurosci.* 31:366–375; *J. Neurosci. Res.* 84:1692–1702). In the present work, effects of BDNF on BF OLG progenitor cells (OPCs) were examined. BDNF increased DNA synthesis of OPCs, as assessed by thymidine and bro-modeoxyuridine incorporation. Effects of BDNF on DNA synthesis were mediated through the trkB receptor and not the p75 receptor, as shown by inhibitors that block neurotrophin binding to the receptors and by the phosphorylation of trkB. TrkB can activate the mitogenactivated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3-K), and phospholipase C- γ (PLC- γ) pathways. BDNF elicited the phosphorylation of MAPK and Akt, a kinase downstream of PI3K, but not PLC- γ in OPCs. Through the use of specific inhibitors to the MAPK and PI3-K pathways, it was found that the MAPK pathway was responsible for the effect of BDNF on DNA synthesis. These data indicate that BDNF affects OPC proliferation and development through the mediation of trkB and the MAPK pathway.

Keywords

p75 NTR; trkB; proliferation; MAPK

In the developing brain, oligodendrocyte progenitor cells (OPCs) proliferate, migrate, and differentiate into mature oligodendrocytes (OLGs) capable of myelinating axons (Levine, 1989; Raff, 1989; Miller, 1996). Recently, oligodendrocyte progenitor cells (OPCs) have been identified as an abundant and widespread population in the adult as well as in the developing animal (Dawson, 2003; Dawson et al., 2000). Current research favors the hypothesis that these OPCs in the adult brain are able to proliferate and differentiate into myelinating OLGs as in development (Gensert and Goldman, 1997; Keirstead et al., 1998; Cenci di Bello et al., 1999; Franklin, 2002). Therefore, with increased understanding of OPCs as therapeutic targets for disease, it may be possible to enhance the ability of remyelination.

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Development of oligodendrocytes is controlled by extracellular signals, which guide the lineage cells through several stages. This course of development has been characterized in vitro, and progression through each stage requires signaling from factors including hormones, growth factors, and cytokines. Of interest to us has been the role that neurotrophins play in OPC development. This family of factors includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Neurotrophins bind and activate two types of receptors, the trk tyrosine kinase receptors, including trkA, trkB, and trkC, and the p75 receptor. Although all neurotrophins bind p75 with similar affinity, NGF is the preferred ligand for trkA, BDNF and NT-4/5 for trkB, and NT-3 for trkC (for reviews see Barbacid, 1994; Chao, 1994; Friedman and Greene, 1999). Oligodendrocytes have been shown to express these neurotrophin receptors (Cohen et al., 1996; Condorelli et al., 1996; Kumar and de Vellis, 1996). Studies in culture have found that neurotrophins affect OPC proliferation (Barres et al., 1994; Robinson and Miller, 1996; McTigue et al., 1998), survival (Cohen et al., 1996; Kahn et al., 1999), and differentiation (Du et al., 2003, 2006a). However, the roles of individual neurotrophins vary in different reports depending on the age of the animal, the brain region under study, and the growth conditions used. For instance, cultured OLGs from the basal forebrain (BF) respond dramatically to BDNF, by differentiating, while cortical OLGs give no response (Du et al., 2003). The BF is of special significance. It is implicated in cognitive deficits of multiple sclerosis and Alzheimer's disease, in which losses, or dysfunction, of OLGs occur. Because of its unique role in the BF, BDNF is of particular interest. This study examines the role of BDNF on OPCs of this brain region and the signaling pathway mediating its effects.

Work in our laboratory has implicated trkB, which possesses a high-affinity binding site for BDNF, as a major receptor mediating differentiation of OLGs (Du et al., 2003, 2006a). Upon BDNF binding the receptor, trkB autophosphorylates and goes on to activate intracellular signaling pathways. Trk receptors activate three major signaling cascades, the mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3-K), and phospholipase C- γ (PLC- γ) pathways (for reviews see Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). Previous work indicated that BDNF's actions through trkB are generally mediated through these pathways (Bulleit and Hsieh, 2000; Han and Holtzman, 2000; Righi et al., 2000; Mizoguchi and Nabekura, 2003).

Here we provide evidence that OPCs of the BF respond to BDNF by increasing DNA synthesis. To investigate further the mechanisms by which BDNF affects OPCs, we examined whether the trkB or p75 receptor was responsible and the signaling pathways mediating this response. BDNF's actions are mediated through the trkB receptor, which stimulates the MAPK pathway, in turn leading to an increase in DNA synthesis.

MATERIALS AND METHODS

Experimental Animals

Pregnant Sprague Dawley rats were obtained from Hilltop Laboratories and housed in clear plastic cages. Food and water were available ad libitum. The animals were managed by the UMDNJ/Robert Wood Johnson Animal Facility, which is accredited by AAALAC. Animal

maintenance, husbandry, transportation, and housing were in compliance with the Laboratory Animal Welfare Act (PL 89–544; PL-91–579). Moreover, our use of animals is in compliance with NIH guidelines (NIH Manual Chapter 4206).

Purified Cell Cultures

Enriched OLG cell cultures were established using the BF of postnatal day 1 (P1) Sprague Dawley rats. The region dissected was posterior to the prelimbic area, anterior to the anterior commissure, medial to the lateral ventricles, and ventral to the corpus callosum. It includes the medial septal nucleus and the diagonal band. Cells were mechanically dissociated and plated in 75-mm flasks coated with poly-D-lysine (0.1 mg/ml). The mixed cell cultures were grown in serum-containing medium (NM-15), consisting of Eagle's minimum essential medium with Earle's salts and L-glutamine (Gibco, Grand Island, NY), heat-inactivated fetal bovine serum (15%), glucose (6 mg/ml), and penicillin-streptomycin (0.5 U/ml and 0.5 µg/ml, respectively). After 12 days of growth, the cells were shaken at 250 rpm for approximately 15 hr to separate the OPCs and microglia from the bottom layer of astrocytes. The OPCs and microglia were then plated on uncoated 100-mm dishes for 1 hr at room temperature. The supernatant containing OLG-lineage cells was collected leaving the microglia bound to the bottom of the plates. Cells were then plated on poly-D-lysine-coated 35-mm dishes in NM-15 at a concentration of 250,000 cells per dish. After 24 hr, cells were switched to serum-free medium (SFM) consisting of 1:1 F-12 nutrient medium and Basal Medium Eagle containing glucose (6 mg/ml), transferrin (100 µg/ml), insulin (25 µg/ml), progesterone (20 nM), putrescine (60 µM), selenium (30 nM), glutamine (6.6 mM), penicillin-streptomycin (0.5 U/ml and 0.5 µg/ml, respectively), triiodothyronine (0.08 µg/ml), and thyroxine (0.5 µM).

Thymidine Incorporation

Incorporation of [³H]thymidine into DNA was assessed by adding [³H]thymidine directly into the culture medium following BDNF treatment. After a 4-hr incubation at 37°C, the cells were harvested and processed for thymidine incorporation by scintillation spectroscopy.

Immunocytochemistry

Bromodeoxyuridine—To assess DNA synthesis, bromodeoxyuridine (BrdU; 0.1 µM) was added to the medium of OPC cultures 20 hr after BDNF treatment. After 4 hr of incubation, cells were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 1 hr at room temperature. The cells were incubated in 2 N HCl to separate DNA strands, then blocked in 30% horse serum/PBS. Exposure to monoclonal anti-BrdU (1:35; Dako, Carpinteria, CA) was followed by biotinylated anti-mouse IgG (1:200; Vector, Burlingame, CA). Positive cells were visualized using the avidin-biotin complex (ABC) method (Vectastain Elite Kit; Vector) and the 3,3'-diaminobenzidine tablet set (DAB; Sigma, St. Louis, MO). The dishes were washed with PBS to terminate the reaction. Cells were analyzed under the light microscope at a magnification of ×250. BrdU-positive cells as well as total cell number were counted in every other field across the diameter of the dish, representing approximately 1% of the total number of cells in the entire dish. Data are expressed as the labeling index

(number of BrdU⁺ cells/total cells). Alternatively, when numbers of cells are indicated in figures, they represent the number of cells counted.

Colabeling for A2B5 and p75 or trkB—Cultures grown for 24 hr in SFM were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 1 hr at room temperature. Cells were blocked in 30% goat serum/0.3% Triton X-100/PBS for 1 hr then incubated for 24 hr in polyclonal anti-trkB (1:250; Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes the full-length trkB receptor or polyclonal anti-p75 (1:1,000; Chemicon, Temecula, CA). After this, cultures were incubated in anti-rabbit IgG (1:200; Vector) for 1 hr then subjected to the ABC-DAB method described above. After incubation in 30% goat serum/0.3% Triton X-100/PBS for 1 hr, A2B5 monoclonal antibody (1:100; Chemicon) was applied for 24 hr at 4°C. Staining for A2B5 was visualized with AlexaFluor 594 anti-mouse IgG (H + L; 1:500; Molecular Probes, Eugene, OR).

Colabeling for p75 and trkB—Cultures grown for 24 hr in SFM were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 1 hr at room temperature. Cells were blocked in 30% horse serum/0.3% Triton X-100/PBS for 1 hr, then incubated for 24 hr in monoclonal anti-p75 (1:100; Chemicon). After this, cultures were incubated in anti-mouse IgG (1:200; Vector) for 1 hr, then subjected to the ABC-DAB method described above. After incubation in 30% goat serum/0.3% Triton X-100/PBS for 1 hr, polyclonal anti-trkB (1:250; Santa Cruz Biotechnology) was applied for 24 hr at 4°C. Staining for trkB was visualized with AlexaFluor 488 anti-rabbit IgG (H + L; 1:1,000; Molecular Probes).

Reagents and Treatments

BDNF (Pepro Tech.) and FGF (Sigma) were both used at 10 ng/ml to treat cell cultures after 4 hr in SFM. For experiments investigating signaling pathways, inhibitors PD98059, U0126, and LY294002 were obtained from Cell Signaling (Beverly, MA). Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and administered to cultures 1 hr prior to BDNF treatment. Control cultures received DMSO as vehicle. To block neurotrophin binding to the p75 receptor, anti-p75 (a gift from M. Chao) was used at 10 µl/ml. Controls were treated with IgG at the same concentration. To block trkB, the trk receptor tyrosine kinase inhibitor K252a (Calbiochem Inc., La Jolla, CA) was used at a concentration of 100 nM. In both cases, the inhibitor was applied for 1 hr prior to BDNF treatment. Control cultures received vehicle DMSO.

Immunoblotting

OPC samples were obtained after 2–20 min of BDNF treatment by scraping them from 35-mm dishes and lysing them in a buffer solution containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 2 mM EGTA, 0.1% SDS, 1% CHAPS, 0.5% NP-40, 1% Triton X-100, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 20 µg/ml soybean trypsin inhibitor, 50 mM NaF, 0.5 µM microcystin-LR, 0.5 mM Na₃VO₄, and 1 mM PMSF. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein for each sample were loaded onto the gel, and electrophoresis was performed on 4–12% (PLC-γ) or 10–20% (MAPK, AKT) Tris-glycine gels (Invitrogen, Carlsbad, CA) for optimal separation. After this procedure, proteins were transferred to PVDF membranes

using a semidry transferring system. The membrane was blocked in 3% bovine serum albumin, then incubated in the appropriate antibody (1:1,000; Cell Signaling) at 4°C overnight. Subsequently, the secondary antibody, horseradish peroxidase-linked IgG (anti-rabbit; Amersham, Arlington Heights, IL) was added at 1:3,000 for 1 hr at room temperature. Phosphorylated protein bands were then visualized using the enhanced chemiluminescence system (Amersham). The same membranes were stripped and reprobed with antibody recognizing both phosphorylated and unphosphorylated forms of the protein to normalize the results.

For immunoprecipitations, cell lysate was mixed overnight at 4°C with anti-trkB, a polyclonal antibody against the extracellular domain (QCB Inc.) and protein A-Sepharose 6MB (100 µl/tube). After centrifugation (10,000g for 5 min at 4°C), the Sepharose-antigen-antibody complexes were washed with buffer (50 mM Tris-HCl, pH 8.1, containing 150 mM NaCl, 1% Triton X-100, 1% CHAPS, 0.5% Nonidet P-40, 0.1% SDS, and 1 mM PMSF) three times. The immunoprecipitates were solubilized and boiled in 40 µl sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002 bromophenol blue) for 5 min and then subjected to electrophoresis. A 4–12% Tris-glycine gradient gel (Invitrogen) was used to obtain optimal separation. Proteins were transferred to PVDF membranes before blocking with BSA. The membrane was probed with antiphosphotyrosine (PY20) to determine levels of phosphorylation. The same membranes were then stripped and reprobed with the trkB antibodies used above (1:1,000) to determine the total amount of receptor protein for normalization. The data of the Western blot experiments were analyzed with Universal Hood Gel Documentation Systems and Quantity One V4.2.1 software (Bio-Rad, Hercules, CA).

RESULTS

Previous studies indicate that BDNF influences differentiating OLGs by increasing the number of MBP⁺ cells as well as expression of PLP, MAG, and MBP protein (Du et al., 2003, 2006a). In these experiments, effects of BDNF are examined in proliferating oligodendrocyte progenitors.

BDNF Increases DNA Synthesis in OPCs

Enriched cultures of OPCs were grown in serum-containing media for 24 hr and then switched to SFM and treated for 1 day with BDNF (10 ng/ml). Thymidine or BrdU was added during the last 4 hr of the 24-hr period, after which the amount of thymidine and BrdU incorporation was determined. BDNF significantly increased the number of cells entering the S phase as determined by thymidine incorporation (Fig. 1A) or monitoring the labeling index (BrdU⁺ cells/total cells; Fig. 1B,C). No change in total cell number was seen in these experiments performed in a nonproliferating serum-free environment (Fig. 1D). However, when the OLGs were grown in a proliferative environment (i.e., containing FGF-2), the total cell numbers increased after BDNF treatment (Fig. 1E).

Effects on BrdU Incorporation are Mediated Through the TrkB Receptor and Not p75

In the peripheral nervous system, BDNF enhances myelin formation and inhibits Schwann cell migration through the p75 receptor (Tolwani et al., 2004; Yamauchi et al., 2004). In the central nervous system, BDNF mediates its effect on differentiation of OLGs through the trkB receptor (Du et al., 2006a). To investigate receptors influencing OPCs in response to BDNF, initial studies evaluated whether OPCs have receptors for BDNF. Immunocytochemical techniques were used to colocalize each of these receptors to A2B5+ OPCs. Subpopulations of OPCs, identified by A2B5 staining exhibited p75 and trkB receptors (Fig. 2A).

To define the receptor mediating BDNF actions, inhibitors were used to block neurotrophin binding to either of these receptors. To determine effects of p75, cells were treated with BDNF with or without a p75 blocking antibody, and BrdU incorporation was assessed. Blocking BDNF binding to p75 did not alter the ability of BDNF to increase BrdU+ cells, suggesting that the p75 receptor is not mediating BDNF actions (Fig. 3A). On the other hand, when the trk tyrosine kinase inhibitor K252a (100 nM) was applied at a concentration shown by others to inhibit trk receptors (Knusel and Hefti, 1992), the drug was able to block the increase in BrdU+ cells in response to BDNF (Fig. 3B). These data suggest that trkB is responsible for mediating BDNF's action.

It is possible that the lack of contribution of p75 to the BDNF effect may be due to a lack of cells coexpressing trkB and p75. To address this possibility, cultures were colabeled with antibodies to trkB and p75. TrkB was colocalized to subpopulations of p75+ cells but was also localized to p75-negative cells (see Fig. 2B). However, when numbers of cells coexpressing trkB and p75 were compared with those expressing trkB alone, eight times the number of cells coexpressed trkB and p75 (42.7% of total cells) as opposed to those expressing trkB alone (5.4% of total cells), suggesting that the lack of effect on proliferation in the presence of p75 blockade is not due to a lack of colocalization of the trkB and p75 receptors (Table I).

To confirm mediation through trkB, the activation of the trkB receptor by BDNF was assessed by Western blot. After treatment with BDNF for 5 min, cells were collected and immunoprecipitated with an antibody against the tyrosine kinase domain of trkB before subjection to electrophoresis. The blot was then probed with PY20 antiphosphotyrosine antibody to determine the level of trkB phosphorylation. Total trkB protein levels were monitored on the same blot to normalize the results. Stimulation of the BF OPCs with BDNF elicited an increase in trkB phosphorylation (Fig. 4A,B). Together with the previous findings, this result indicates that the BDNF effects on DNA synthesis in OPCs are mediated through trkB.

Three major pathways have been demonstrated to mediate effects of BDNF through trkB. These are the MAPK, PI3-K, and PLC- γ signaling cascades. To define the signaling cascade underlying BDNF actions, these three pathways were examined.

The MAPK Pathway Plays a Role in BDNF's Effect on DNA Synthesis

The first pathway examined as potentially activated by BDNF was the MAPK pathway. Previous work in our laboratory indicated that this pathway is activated and plays a role in OLG differentiation (Du et al., 2006b). To determine whether it also plays a role in DNA synthesis, experiments were performed to examine pathway activation. After 4 hr in SFM, cultures were treated with BDNF or vehicle. Phosphorylation levels of MAPK were monitored by using a specific antiphospho-p44/42 MAPK antibody. The membrane was then stripped and probed with an antibody to MAPK protein for normalization (Fig. 5A). Phosphorylated MAPK is increased relative to total protein levels in BDNF-treated cultures compared with control after 5 min of BDNF stimulation (Fig. 5B), suggesting that this pathway is activated by BDNF.

To determine whether the activation of the MAPK pathway underlies the increase in DNA synthesis elicited by BDNF, specific inhibitors of the pathway were used. Cultures were exposed to the inhibitors U0126 (2 μ M) or PD98059 (50 μ M), which block activation of MEK1/2, the upstream kinase of MAPK. Treatment with U0126 blocked effects of BDNF on DNA synthesis (Fig. 5C). Similar results were noted with the drug PD98059 (data not shown). This experiment suggests that the MAPK pathway mediates BDNF actions. When number of cells was evaluated, there was no difference among the groups [cell numbers = 785 ± 164 (control), 715 ± 194 (BDNF), 835 ± 238 (U0126), 806 ± 210 (U0126 + BDNF)], suggesting that inhibition of the MAPK pathway specifically blocked BDNF elicited DNA synthesis and did not influence cell number.

The PI3-K Pathway Is Not Involved in BDNF's Effect on DNA Synthesis

To investigate whether other pathways mediate BDNF actions, the PI3-K signaling cascade was evaluated. After 4 hr in SFM, cultures were treated with BDNF for 5 min, lysed, and collected for Western blot analysis. Activation of the pathway was determined by using an antibody to phosphorylated Akt, a kinase activated downstream in the pathway. The membrane was then stripped and probed with an antibody to Akt protein to normalize the results (Fig. 6A). The densitometric analysis shows an increased activation of phosphorylated Akt elicited by BDNF compared with control (Fig. 6B). To determine whether activation has any effect on DNA synthesis in OPCs, LY294002, an inhibitor of Akt activation, was used to block PI3-K prior to screening for BrdU incorporation. No changes in the BDNF-elicited increase in BrdU incorporation relative to control were observed (Fig. 6C), suggesting that this pathway does not mediate actions of BDNF on this process. It was possible that the dose of LY294002 (10 μ M) was not optimal for inhibition of PI3-K. The ability of LY294002 at this dose to inhibit Akt phosphorylation was therefore evaluated. LY294002 completely blocked the activation (Fig. 6B). No changes in cell number were detected after 24 hr [total cells = $1,301 \pm 322$ (control), $1,359 \pm 453$ (BDNF), $1,079 \pm 248$ (LY294002), $1,402 \pm 544$ (LY294002 + BDNF)].

To confirm results observed using LY294002, another inhibitor of Akt activation, wortmannin (50 nM), was used to block PI3-K prior to screening for BrdU incorporation. As seen with LY294002, no changes in BrdU incorporation were seen following wortmannin treatment (Fig. 6F). Wortmannin at a dose of 50 nM completely blocked the phosphorylation

of Akt (Fig. 6 D,E), supporting the observation that the PI3-K pathway does not mediate effects of BDNF on DNA synthesis. Again, none of the PI3-K manipulations resulted in changes in cell number after 24 hr [total cells = 230 ± 50 (control), 239 ± 46 (BDNF), 188 ± 40 (wortmannin), 233 ± 48 (wortmannin + BDNF)], suggesting that the effects observed are due solely to changes in BrdU incorporation.

The PLC- γ Pathway Is Not Involved in BDNF's Effect on DNA Synthesis

The last pathway examined was the PLC- γ signaling cascade. After 4 hr in SFM, cultures were treated with BDNF for 2 min, then lysed and collected for Western blot analysis. Activation of the pathway was determined by using a specific antibody to phosphorylated PLC- γ . The membrane was then stripped and probed with an antibody recognizing total PLC- γ levels to normalize the results. No increase in phosphorylation levels of PLC- γ was detected after treatment with BDNF (Fig. 7A,B), suggesting that BDNF does not activate this pathway in these cells.

DISCUSSION

Previous studies in our laboratory indicate that BDNF, through mediation of trkB and the MAP kinase pathway, enhances OLG differentiation (Du et al., 2006a,b). The present work suggests that BDNF, through the same receptor and signaling pathway, elicits an increase in DNA synthesis and in a proliferating environment an increase in populations of OPCs. Investigation into the three major pathways activated by trkB, MAPK, PI3-K, and PLC- γ , determined that the MAPK and PI3K pathways exhibited increased activation, whereas the PLC- γ pathway did not. Moreover, inhibitors to the MAPK pathway alone were able to decrease significantly the number of BrdU-positive cells compared with control, suggesting that this pathway plays a role in promoting proliferation.

BDNF, then, through the mediation of these pathways, joins a number of other factors that affect OPC proliferation and development. For example, platelet-derived growth factor (PDGF), FGF-2, neuregulin, and the neurotrophins NGF and NT-3 in combination with PDGF increase progenitor proliferation (Pringle et al., 1989; McKinnon et al., 1991; Althaus et al., 1992; Barres et al., 1994; Canoll et al., 1996; Cohen et al., 1996; Engel and Wolswijk, 1996; Baron et al., 2000). These molecules appear to be effective in culture (Pringle et al., 1989; McKinnon et al., 1991; Althaus et al., 1992; Barres et al., 1994; Canoll et al., 1996; Baron et al., 2000), in vivo (Calver et al., 1998), and after a lesion (Murtie et al., 2005). The pathways mediating these events, in some cases, include the MAPK pathway. NT-3, PDGF, and FGF-2 are reported to employ this pathway to effect mitogenesis (Baron et al., 2000; Johnson et al., 2000). On the other hand, it is also reported that PDGF makes use of both the PI3-K and the PLC- γ pathways to induce proliferation (McKinnon et al., 2005). Clearly, the OPCs are able to respond to multiple environmental signals to effect the same process.

Interestingly, effects of BDNF appear to be limited to DNA synthesis and do not result in increases in cell number after 24 hr. FGF-2 appears to be necessary to elicit such increases. This may be due to an inability of BDNF alone to support progression through the cell cycle. As is being shown in multiple systems, cell cycle progression may be specifically affected by the presence of individual factors or combinations of such factors. For example,

interferon- γ increases delay of cells in G2 and inhibits progression to mitosis (Chew et al., 2005). On the other hand, FGF-2 in combination with IGF-1 enhances progression to G2/M. Interestingly, this progression elicited by the two-factor combination is greater than that observed with either factor alone (Frederick and Wood, 2004). Therefore, multiple factors must control the cell cycle, and the coordinate actions of several factors may be necessary to complete the complex process of proliferation as well as survival and subsequent differentiation (Barres et al., 1994; Raff et al., 1994).

Before closing it is important to make one further point. Our studies used mature (14-kDa) BDNF to elicit effects on DNA synthesis. Recently, it is being appreciated that BDNF may be released from cells as multiple isoforms that include the mature 14-kDa form as well as higher molecular weight forms of 28 and 32 kDa (Lee et al., 2001). Whereas the 14-kDa form preferentially binds to the trkB receptor, the higher forms preferentially bind to the p75 receptor (Fayard et al., 2005), and these forms may have distinct actions on target cells (Woo et al., 2005). We have not addressed the roles of these isoforms on OPC proliferation. This will be a subject of future investigation and add further to our understanding the pathways and molecules that underlie effects of BDNF on OPCs.

The study of these and other molecules is critical because of the role that OPCs may play in the developing and adult brain. In demyelinating diseases such as multiple sclerosis, oligodendrocytes die and progenitor cells attempt to remyelinate the damaged axon tracts. The cells are only partially successful. To determine how to optimize this process, three important stages of development, proliferation, survival, and differentiation, must be examined. By studying the pathways leading to proliferation, in the future therapeutic agents may be found that can strictly stimulate pathways that regulate the expansion of OPCs and lead to their repopulation of damaged axons.

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Fig. 1.

BDNF increases DNA synthesis in OPCs. **A:** Cells were treated with BDNF (10 ng/ml) for 24 hr and pulsed with [3 H]thymidine for the last 4 hr. The cells were harvested and processed for incorporation by scintillation spectroscopy. **B:** Cells were treated with BDNF (10 ng/ml) for 24 hr and BrdU for 4 hr and then were fixed and stained for BrdU incorporation (arrows). **C:** Labeling index (BrdU $^{+}$ cells/total cells) in BDNF-treated cultures compared with control, expressed as percentage control. **D:** Total number of cells was not affected by BDNF. **E:** FGF (10 ng/ml) increases total cell numbers. For all experiments, the figure represents one experiment that was replicated three times with similar results. Each group had at least three culture dishes. *Significantly different from control at $P < 0.01$. **Significantly different from FGF alone at $P < 0.05$. Data were analyzed using Student's t -test or ANOVA, followed by Fisher's protected least significant difference post hoc test as necessary.

Fig. 2.

A: TrkB and p75 are present in subsets of A2B5+ oligodendrocytes. Enriched oligodendrocyte cultures were grown for 24 hr in SFM and processed for immunocytochemistry. Cells were then stained for trkB or p75 (visualized with DAB) and A2B5 (visualized with AlexaFluor; red). Arrows indicate cells positive for receptor and A2B5; arrowheads indicate cells positive for only one antigen. **B:** TrkB and p75 are colocalized in a subset of oligodendrocyte precursors. Enriched oligodendrocyte cultures were grown for 24 hr in SFM and processed for immunocytochemistry. Cells were then

stained for p75 (visualized with DAB) and trkB (visualized with AlexaFluor; green). Arrows indicate cells positive for both receptors; arrowheads indicate cells positive for only one antigen. Scale bar = 100 μ M.

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Fig. 3.

A: The p75 blocking antibody does not inhibit BrdU incorporation in the presence of BDNF. BDNF treatment elicited a significant increase in the labeling index compared with control values. Purified OPCs were treated with 10 ng/ml of BDNF $\pm$ p75 antibody for 24 hr. **B:** The trk tyrosine kinase inhibitor K252a blocks BDNF's effect on DNA synthesis. Cells were treated with BDNF (10 ng/ml) with or without K252a (100 nM) for 24 hr. For all experiments, the figure represents one experiment that was replicated three times with similar results. *Significantly different from control at $P = 0.05$. Data were analyzed using

Student's t -test or ANOVA, followed by Fisher's protected least significant difference post hoc test.

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Fig. 4. TrkB in BF OPCs is activated by treatment of BDNF. Four hours after changing to SFM, OPCs were treated with BDNF (10 ng/ml) for 5 min before proteins were harvested. **A:** Immunoprecipitation and Western blots were used to examine the phosphorylation and total protein levels of trkB. **B:** Levels of phosphorylation relative to trkB protein were calculated in one experiment, and the result is shown as percentage control trkB phosphorylation/trkB protein level. A second experiment yielded similar results.

Fig. 5. BDNF increases BrdU incorporation in OPCs through MAPK. Phosphorylation levels of MAPK are increased with BDNF. BrdU incorporation is blocked by the MEK1/2 inhibitor U0126 (2 μ M). **A:** Western blot of control and BDNF-treated cultures probed with antibody to phosphorylated MAPK, then stripped and reprobed for total MAPK protein. **B:** Densitometric analysis of MAPK activation normalized to total MAPK protein derived from three independent experiments. **C:** Labeling index for control and BDNF-treated cultures expressed as percentage control. Data are derived from three separate experiments. Cells were grown for 24 hr in the presence or absence of BDNF $\pm$ U0126 (2 μ M) and pulsed with

BrdU for the final 4 hr. *Significantly different from control at $P < 0.05$. Data were analyzed using Student's *t*-test or ANOVA, followed by Fisher's protected least significant difference post hoc test.

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Fig. 6.

Phosphorylation levels of Akt are increased by BDNF and blocked to control levels by the PI3 kinase inhibitors LY294002 (LY; 10 μ M) or wortmannin (WT; 50 nM). **A:** Western blot of control and BDNF-treated cultures with or without LY probed with an antibody to phosphorylated Akt and then stripped and reprobed for total Akt kinase protein. **B:** Densitometric analysis of Akt activation normalized to total Akt protein derived from four independent experiments. **C:** Labeling index for control and BDNF-treated cultures, with or without LY, expressed as percentage control. Data derived from three separate experiments. Cells were grown for 24 hr in the presence or absence of BDNF $\pm$ LY (10 μ M) and pulsed

with BrdU for the final 4 hr. **D:** Western blot of control and BDNF treated cultures with or without WT probed with an antibody to phosphorylated Akt and then stripped and reprobed for total Akt kinase protein. **E:** Densitometric analysis of Akt activation normalized to total Akt protein derived from three independent experiments. **F:** Labeling index for control and BDNF-treated cultures with or without WT expressed as percentage control. Data derived from three separate experiments. Cells were grown for 24 hr in the presence or absence of BDNF $\pm$ WT (50 nM) and pulsed with BrdU for the final 4 hr. *Significantly different from control at $P < 0.05$. Data were analyzed using Student's *t*-test or ANOVA, followed by Fisher's protected least significant difference post hoc test.

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Fig. 7. BDNF has no effect on PLC- γ phosphorylation. **A:** Western blot of control and BDNF-treated cultures probed with antibody to phosphorylated PLC- γ and then stripped and reprobed for total PLC- γ protein. **B:** Densitometric analysis of PLC- γ activation normalized to total PLC- γ protein derived from three independent experiments. Data are expressed as mean $\pm$ SEM. Data were analyzed using Student's *t*-test.

TABLE I

p75 and TrkB Receptor Expression in Oligodendrocytes *

	p75+ /TrkB+	p75+ Alone	TrkB+ alone	p75-/trkB-
Total cells (%)	42.7	33	5.4	18.9

* Control cultures grown for 24 hr in serum-free media were stained with antibodies for p75 (Chemicon; 1:200) and TrkB (Santa Cruz Biotechnology; 1:250). Double-labeled cells, single-labeled cells, and negative cells were counted at $\times 400$ and are expressed as the percentage of total cells counted.