BDNF Enhances the Differentiation but Not the Survival of CNS Stem Cell-Derived Neuronal Precursors

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We have previously reported the isolation of an EGF-responsive precursor from the embryonic and adult mouse striatum. This precursor exhibits self renewal and the ability to produce a sphere of undifferentiated cells which can be induced to differentiate into neurons and glia. RT-PCR analysis of these spheres of undifferentiated cells revealed the expression of mRNA for the *trkB* neurotrophin receptor, both with and without the catalytic domain, and little or no expression of *trkA* or *trkC*. We examined the actions of BDNF on the fate of EGF-generated neural precursors. Ten days after a one-time exposure to BDNF, single EGF-generated spheres showed a twofold increase in neuron number and a marked enhancement in neurite outgrowth. Examination of neuronal nuclei with immunochemical probes for *c-fos* and bromodeoxyuridine revealed that the actions of BDNF were directly upon neuronal cells and did not involve division of neuronal precursors. The twofold increase in neuronal number due to BDNF, observed after 10 d *in vitro*, was significantly reduced after 21 d *in vitro* and was not apparent at 27 d *in vitro*. Quantitative analyses revealed that while repeated application of BDNF did not prevent the loss of neuron number over time, it did result in a significant increase in neurite numbers. Moreover, delayed addition of BDNF mimicked the increase in neuronal numbers seen when BDNF was present throughout. These BDNF actions did not appear to involve the enhancement of a novel neuronal phenotype, with all effects being due to increase in the numbers and neurite outgrowth of neurons that colocalize GABA and substance P. These findings suggest that BDNF markedly enhances the antigenic and morphologic differentiation of EGF-generated neuronal precursors. BDNF alone does not appear to act as a survival factor for neuronal precursors nor is it sufficient for preventing their death over time.

[Key words: brain-derived neurotrophic factor, *trkB*, stem cells, neuronal precursors, neuronal differentiation, neurite outgrowth]

Cellular diversity in the mammalian CNS is generated from a small number of precursor cells in the neural ectoderm. While the existence of multipotent precursors for neurons and glia has been described both *in vitro* (Raff, 1989; Temple, 1989; Williams et al., 1991; Kilpatrick and Bartlett, 1993) and *in vivo* (Turner and Cepko, 1987; Frederiksen and McKay, 1988; Galileo et al., 1990), the signals that regulate their proliferation, differentiation and subsequent survival are less well understood. The neurotrophins, a family of epigenetic factors that influence neural development, include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin 6 (NT-6) (Leibrock et al., 1989; Hohn et al., 1990; Maisonpierre et al., 1990; Berkemeier et al., 1991; Hallbook et al., 1991; Gotz et al., 1994). These factors are small proteins, which share at least 50% sequence homology, regulate proliferation of neuronal precursors, and enhance the survival and differentiation of postmitotic neurons (reviewed in Davies, 1994). Signal transducing receptors for neurotrophins, collectively referred to as *trks*, are also a structurally related group of proteins (Chao, 1992; Barbacid, 1994). These receptors fall into a family of protein tyrosine kinase receptors, consisting of an extracellular binding domain, a transmembrane region and an intracellular catalytic kinase domain (Klein et al., 1989; Lamballe et al., 1991). Although each receptor exhibits high affinity binding of its respective ligand, for example *trkB* encodes a functional receptor for BDNF (Klein et al., 1991; Soppet et al., 1991; Squinto et al., 1991), multiple interactions exist between the *trks* and the neurotrophins (reviewed in Barbacid, 1994). The spatiotemporal expression of the different neurotrophins and their receptors, in the embryo and the adult CNS, suggests that these molecules play specific roles throughout development.

Consistent with the widespread distribution of *trkB* in the PNS and CNS (Klein et al., 1990), BDNF has been reported to have survival and/or differentiation actions on a number of neuronal populations *in vitro*. BDNF promotes survival and neurite outgrowth of cultured neural crest-derived and placode-derived sensory neurons and parasympathetic nodose ganglion neurons (Lindsay et al., 1985; Davies et al., 1986) but not sympathetic neurons (Lindsay and Rohrer, 1985). In the CNS, BDNF supports the survival and phenotypic differentiation of basal forebrain cholinergic neurons (Alderson et al., 1990; Knusel et al., 1991) and enhances the survival of cultured dopaminergic neurons (Hyman et al., 1991, 1994; Knusel et al., 1991), cerebellar granule cells (Segal et al., 1992; Lindholm et al., 1993), retinal

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ganglion cells (Johnson et al., 1986; Rodriguez-Tebar et al., 1989) and motor neurons (Wong et al., 1993). In addition to promoting survival, BDNF also regulates the expression of phenotypic markers of cultured GABAergic neurons of the developing ventral mesencephalon and striatum (Knusel et al., 1991; Ventimiglia et al., 1993; Hyman et al., 1994) and that of calbindin-containing neurons of striatum and the hippocampus (Collazo et al., 1992; Ventimiglia et al., 1993). Recent findings indicate that BDNF is able to elevate cellular GABA content of cultured striatal neurons (Mizuno et al., 1994) and increase the numbers of neurons expressing calbindin or AChE (Ip et al., 1993), without affecting the survival of these cells. Thus, although considerable evidence has accumulated, suggesting a role for BDNF in the development of central neurons, its precise action, for example, survival versus differentiation, remains unclear.

We have reported the isolation, from the embryonic and adult mouse striatum, of an epidermal growth factor (EGF)-responsive CNS precursor cell (Reynolds and Weiss, 1992; Reynolds et al., 1992) that exhibits self renewal and the ability to generate neurons and glia. These *in vitro* properties and the ability to participate in repopulation in the adult CNS *in vivo* (Morshead et al., 1994), suggest that this EGF-responsive precursor is a stem cell. These stem cells proliferate in response to EGF and form clusters (spheres), which lift off the substrate and float in suspension where they continue to proliferate. At 6–7 d *in vitro* (DIV), the majority of the cells within these spheres express immunoreactivity for nestin, an intermediate filament found in undifferentiated cells of the CNS, but they do not express any of the antigens characteristic of mature CNS cells. Clonal analysis of individual cells within the EGF-generated spheres reveals that some of the cells produced can generate new spheres (Reynolds and Weiss, unpublished observations), while others respond to other mitogens, such as basic fibroblast growth factor (bFGF) and undergo limited proliferation to generate neurons and glia (Vescovi et al., 1993). In the latter study, while bFGF was able to induce the proliferation of neuronal progenitor cells, these cells required additional factors for their terminal differentiation. In order to further our understanding of the steps between stem cell proliferation and the production and phenotypic determination of neurons, we asked whether the stem cell progeny expressed any of the *trk* receptors. Identification of the *trkB* receptor on these cells prompted us to examine the actions of BDNF on EGF-generated neuronal precursors. Our results suggest that BDNF acts as a differentiation factor for neuronal precursors arising from the EGF-responsive stem cell. In addition, our findings argue against BDNF being sufficient to ensure the long term survival of these newly generated neurons.

Materials and Methods

Primary cell culture. Striata were removed from 14 d old CD1 albino mouse embryos (Charles River) and mechanically dissociated in culture media with a fire-narrowed Pasteur pipette. The culture media was composed of DMEM/F12 (1:1) (GIBCO), glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), and HEPES buffer. A hormone and salt mixture including insulin (25 µg/ml), transferrin (100 µg/ml), progesterone (20 nM), putrescine (60 nM), and selenium chloride (30 nM) was used as supplement (all from Sigma except glutamine; GIBCO). Cells were plated at a density of 200,000 cells/ml in Corning T75 (GIBCO/BRL) culture flasks in the above media in the presence of 20 ng/ml EGF (purified from mouse submaxillary; Collaborative Research). After 6 DIV, floating clusters of cells (spheres) were centrifuged (400 rpm) and the EGF-containing media removed. The pellet was mechanically dissociated and reseeded in fresh EGF-containing media at 50,000

cells/ml, until secondary spheres were generated. All spheres used for examining the actions of BDNF were passaged at least once.

Reverse transcription, PCR, and sequencing for *trks*. Total RNA from EGF-generated spheres was prepared utilizing the single-phase phenol: guanidine isothiocyanate reagent Trizol according to manufacturer's instructions (BRL). Cells were pelleted at $500 \times g$ and lysed directly in the reagent (0.2 ml/ 10^6 cells). The organic and aqueous phases were separated by addition of 0.2 volumes of chloroform and centrifugation at $3500 \times g$. Total RNA was precipitated from the aqueous phase with addition of an equal volume of isopropanol and incubation at -20°C overnight. The RNA pellet was redissolved in water and RNA concentration was determined by absorbance at 260 nm. The RNA was reprecipitated with addition of 2 volumes ethanol/50 mM NH_4Ac , followed by overnight incubation at -20°C and resuspended in water for direct use in reverse transcriptase reactions. First strand cDNA was made utilizing Superscript reverse transcriptase following the method as outlined by the manufacturer (BRL). Briefly, 1 µg of total RNA and 0.5 µg oligo dT was heated to 65°C in 12 µl of H_2O and quickly chilled on ice. The annealed mRNA:oligo dT complexes were incubated at 42°C for 2.5 hr with 200 U of reverse transcriptase in 20 µl total volume of 0.5 mM dNTPs; 50 mM Tris pH 8.3; 75 mM KCl; 3 mM MgCl_2 , and 10 mM DTT. The reaction mix was heat inactivated (65°C for 15 min) and diluted to 100 µl with H_2O . The diluted reaction mix was used directly in PCR reactions. Primers specific for individual members of the *trk* receptor family were designed from published sequence data as follows: (1) *trkA*, accession #M85214 (*Rattus rattus*), *trkA*(2253) = 5'-ggtagcagctctccaactgagg-3', *trkA*(2456c) = 5'-ccagaacgtccaggtaactcggtg-3', 204 bp amplified product; (2) *trk B* full length catalytic form, accession #X17647 (*Mus spretus*), *trkB*+kd(1806) = 5'-tctatgccgtgtgtgattgc-3', *trkB*+kd(2228c) = 5'-gcgtcttcacagccaccaggat-3', 423 bp amplified product; (3) *trk B* truncated noncatalytic form, accession #M33385 (*Mus musculus*), *trkB*-kd(1745) = 5'-aagcgagctgttgactatagc-3', *trkB*-kd(2145c) = 5'-gtgcacttagacatagctatgactt-3', 397 bp amplified product; (4) *trk C*, accession #L14447 (*Rattus norvegicus*), *trkC*(2384) = 5'-catgttccagctctcttaacacag-3', *trkC*(2606c) = 5'-accagtcaccactagccaa-gaatg-3', 223 bp amplified product; (5) beta-actin, accession #X03672 (*Mus musculus*), actin(182) = 5'-cgtggccgcctaggcaccac-3', actin(424c) = 5'-ttggccttaggttcagggggg-3', 243 bp amplified product. Numbers in brackets refer to the position of the primer in the nucleotide sequence, "c" denotes the complementary strand. Polymerase chain reaction analyses were performed in a final volume of 50 µl containing 25 nmol of each primer pair, 2.5 µl of the reverse transcription reaction, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , 200 µM each of the four dNTPs and 1 U of taq DNA polymerase (Perkin Elmer). After a denaturation step of 5 min at 95°C , amplifications were carried out for 25 cycles of $94^\circ\text{C}/30$ sec, $60^\circ\text{C}/30$ sec, and $72^\circ\text{C}/1$ min. Final products were analyzed on a 2% agarose/TAE gel. PCR amplification products were excised out of the agarose gel and isolated utilizing spin columns according to the manufacturer's protocol (Qiagen). The purified fragments were sequenced directly utilizing specific primers and the dsDNA cycle sequencing system according to manufacturer's instructions (BRL).

Plating spheres to examine the actions of BDNF. Sterile glass coverslips in 24-well plates (Nunc) were incubated overnight at 37°C in a solution of polyornithine (15 µg/ml; Sigma) in dd H_2O . Twenty-four hours later, excess polyornithine was removed and the coverslips were rinsed three times (5 min each) with PBS. Media was added to plates and allowed to equilibrate at 37°C . EGF-generated spheres were rinsed free of EGF by centrifuging at 400 rpm for 5 min, aspirating the EGF-containing supernatant, and replacing quickly with media. Resuspended spheres were then transferred into 35 mm petri dishes and also allowed to equilibrate at 37°C before plating. Four to five individual spheres were transferred by micro manipulation onto the polyornithine coated coverslips. The spheres were cultured in 1 ml of media containing 1% FBS (UBI, Lake Placid, NY) in the absence or presence of human recombinant BDNF (10 ng/ml) (generously supplied by Dr. M. Barbacid, Bristol Meyers Squibb, Princeton, NJ). This concentration of BDNF has been demonstrated to enhance the *in vitro* survival of retinal ganglion cells (Rodriguez-Tebar et al., 1989) and septal cholinergic neurons (Alderson et al., 1990), and to enhance both the survival and neurite outgrowth of cultured cerebellar granule cells (Lindholm et al., 1993). BDNF was added to the culture media only at the time of initial plating unless otherwise indicated. Spheres were cultured for different lengths of time depending on the experiment, typically 10 DIV.

Antibodies. Mouse monoclonal antibodies and rabbit polyclonal an-

tisera directed against neuronal and glial antigens and neurotransmitter phenotypes were used as primary antibodies for indirect immunofluorescence. Polyclonal anti-neuron specific enolase (NSE) (1:1000) was kindly provided by Dr. R. Hawkes (University of Calgary, AB). Polyclonal anti-glial fibrillary acidic protein (GFAP, 1:1000) was a gift from Dr. L. Eng (Stanford University School of Medicine, CA). Polyclonal antisera directed against substance-P (1:500), 5-HT (1:1000), met-enkephalin (1:1000), somatostatin (1:100), and neuropeptide-Y (1:100) were purchased from Incstar (Stillwater, MN). Polyclonal anti- γ -amino butyric acid (GABA, 1:5000) and monoclonal anti- β -tubulin (Type III; 1:1000) were purchased from Sigma. Polyclonal antisera directed against tyrosine hydroxylase (1:750) and glutamate (1:5000) were purchased from Pel-Freez (Rogers, AR) and Arnel (New York, NY), respectively. Monoclonal antibodies against GFAP, (1:100), microtubule associated protein-2 (MAP-2, 1:400), and O4 (1:10) were obtained from Boehringer Mannheim, (Laval, Quebec). Anti-bromodeoxyuridine (BrdU, 1:5), used in proliferation assays was obtained from Amersham (Oakville, Ontario) and polyclonal antiserum to the immediate-early gene product *c-fos* (1F6; 1:100), was a gift from Dr. K. Riabowal (University of Calgary, Calgary, AB).

Secondary antibodies raised in goat against mouse and rabbit immunoglobulins, conjugated to the fluorophore rhodamine isothiocyanate (RITC, 1:200) or fluorescein isothiocyanate (FITC, 1:100), were purchased from Jackson Immunochemicals (Westgrove, PA). Alkaline phosphatase conjugated goat anti-rabbit IgG (1:100) was purchased from GIBCO.

Immunocytochemistry. After the appropriate number of DIV (depending on the experiment), spheres were fixed with 4% paraformaldehyde (plus 0.1% glutaraldehyde for GABA immunostaining) for 20 min, rinsed three times in PBS (10 min. each), and incubated with primary antibodies in PBS containing 10% normal goat FBS for 2 hr at 37°C. Permeabilization for intracellular antigens was achieved by adding 0.3% Triton X-100 (BDH, Toronto, Ontario) to primary antibody solutions. For dual-labeling experiments, primary antibodies generated from different species were added together. Following incubation coverslips were rinsed three times (10 min each) with PBS and incubated in secondary antibodies for 30 min in the dark at room temperature. Again, for dual labeling, appropriate secondary antibodies were added together. After three final washes in PBS, coverslips were rinsed with double distilled water (dd H₂O) and mounted with FluorSave on glass slides. Fluorescence was detected and photographed with a Nikon Optiphot photomicroscope.

For some experiments, neuronal cell numbers were quantified by using indirect immunocytochemistry with enzyme-linked, rather than fluorescence-linked secondary antibody. The NSE antibody was recognized by incubating the coverslips with goat anti-rabbit IgG linked to alkaline phosphatase for 30 min at room temperature. Staining was developed with a 0.1 M Tris buffer (pH 9.5) containing NaCl (0.1 M), MgCl₂ (5 mM), nitro blue tetrazolium (NBT) (0.3 mg/ml), and bromochloroindolyl phosphate (BCIP) (0.15 mg/ml). The reaction was terminated by rinsing with tap water.

Bromodeoxyuridine (BrdU) labeling and detection. To determine if cells were dividing in the absence or presence of BDNF, BrdU (1 μ M) was added to each of the wells containing spheres (in media with 1% FBS) at the time of plating. BrdU remained in the media for the duration of the culture. At 10 DIV, neuronal and glial proliferation was visualized by dual label immunocytochemistry for BrdU and the neuronal/glial antigens NSE, MAP-2, and GFAP. The identity of the BrdU incorporating cells was further confirmed by morphological examination.

Growth factor-induced expression of *c-fos*. EGF-generated spheres were rinsed free of EGF as described above and resuspended in media containing 1% FBS. A few of these were then transferred onto polyornithine coated glass coverslips in culture plates. At 3 DIV the spheres were exposed to EGF (20 ng/ml) or BDNF (100 ng/ml), for 30–180 min. These coverslips were transferred into 4% paraformaldehyde for fixation, rinsed in PBS three times, and incubated for 45 min at 37°C with *c-fos* antiserum and the neuronal antigen β -tubulin or the glial antigen GFAP. Incubation in the primary antibodies, was followed by incubation in appropriate secondary antibodies and the cellular response to EGF/BDNF stimulation was examined by looking for immunoreactivity for *c-fos* and β -tubulin or GFAP.

Quantification of cell numbers and process outgrowth. Quantification of neuronal numbers was performed double blind, by counting neuronal cell bodies resulting from differentiation within each sphere at time intervals designated by the particular experiment. Neuronal cells were

identified by morphology and their immunoreactivity for NSE, MAP-2 or β -tubulin. The numbers of neurons counted in all the experiments were pooled within each condition and divided by the total number of spheres in that procedure. The resultant mean number of neurons per sphere in each procedure was used in data illustration and analysis. For statistical analysis of the data, the dependent measure was the number of neurons per sphere and the null hypothesis was that the number of neurons per sphere in the different procedures would be the same. χ^2 analyses were carried out to determine significance across entire experiments and to further determine which difference between procedures were significant. Levels of significance were $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Quantification of process outgrowth (in Table 1) was achieved by preparing composites of five spheres in each of the six procedures described. Process outgrowth was quantified 21 DIV and in a manner such that the reader was blind to experimental procedures. Data on process outgrowth was obtained from the same experiments that were carried out for the quantification of neuronal numbers in the previous section. Histochemical immunostaining for NSE was utilized. Photographing consecutive fields of view at high magnification, allowed for resolution sufficient for the quantification of processes extending out from cells around the core of the sphere. The number of neurite intersections at a circle drawn a constant distance of 120 μ m from the center of the sphere for each sphere were counted.

Results

*Cells within the EGF-generated spheres express *trkB**

Reverse transcription-PCR analysis was used to examine the mRNA expression of the *trk* receptor family in EGF-generated spheres of undifferentiated cells. PCR primer pairs specific for beta-actin, *trkA*, the catalytic and noncatalytic forms of *trkB*, and *trkC* were designed from published sequence data and a representative RT-PCR experiment is illustrated in Figure 1. mRNA transcripts for the full-length catalytic and truncated noncatalytic forms of *trkB* were readily detected after 25 cycles of PCR. This result was confirmed in two additional, independent RT-PCR experiments with newly isolated mRNA from EGF-generated spheres. While the expression of *trkA* and *trkC* mRNA is not readily detected in EGF-generated spheres, trace amounts of *trkA* and *trkC* amplified products could be seen if the PCR reactions were driven past 40 cycles (data not shown). The identity of the specific amplified fragments were confirmed by double-strand cycle sequencing with taq polymerase. These data suggest that the catalytic and non-catalytic isoforms of *trkB* are the only members of the neurotrophin receptor family that are transcribed in robust levels in cells within the EGF-generated spheres.

Neuronal cells within the EGF-generated spheres respond to BDNF

The actions of BDNF on EGF-generated spheres was examined by plating spheres on coverslips, rinsed free of EGF and in the presence of 1% fetal bovine serum (FBS), followed by indirect immunocytochemistry after various periods *in vitro*. When examined after 10 d *in vitro* (DIV), both in the absence or presence of BDNF, neurons were identified by their morphologic and antigenic properties (Fig. 2). These neuronal cells possessed relatively small round or pyramidal cell bodies with thin processes. Dual label immunocytochemistry demonstrated that cells with these morphological characteristics expressed the antigens neuron-specific enolase (NSE) and microtubule associated protein-2 (MAP-2) (Fig. 2A,B), as well as β -tubulin (Type III) and the neurotransmitter GABA (Fig. 2C,D). The glial cells within the spheres, astrocytes and oligodendrocytes, were identified by the expression of GFAP and O4 immunoreactivities, respectively (data not shown).

Figure 1. PCR amplifications of neurotrophin receptor mRNA in EGF-generated spheres of undifferentiated cells. First strand cDNA from EGF-generated spheres of undifferentiated cells was amplified utilizing neurotrophin receptor specific primer sets as described in Materials and Methods. *Mr*, 100 bp ladder (BRL); *Act*, β -actin; *trkB*⁺, full length *trkB* containing the catalytic kinase domain; *trkB*⁻, truncated *trkB* lacking the cytoplasmic kinase domain. The subscript c denotes negative control reaction lacking the RT product. Amplified products were run out on a 2% agarose/TAE gel and visualized with ethidium bromide. The figure is printed in reverse contrast for clarity.

In order to determine the phenotype of the cells responding to BDNF, we examined the expression of *c-fos* protein (Greenberg et al., 1985; Curran and Morgan, 1991) in cells within the EGF-generated spheres. In preliminary experiments (data not shown), spheres were rinsed free of EGF, cultured in media containing 1% FBS for 3 DIV, and reexposed to EGF for 30, 60, 90, 120, or 180 min. We expected EGF to induce expression in a large proportion of the cells and this would be useful as a

validation of the assay and a reference to which the BDNF response could be compared. Immunoreactivity to *c-fos* was detected in cells within spheres exposed to EGF for 30 min but cellular nuclei were most intensely and clearly labeled after a 60 min exposure. By 120 min diffuse *c-fos* staining was observed throughout the cell and with no nuclear specificity. When spheres had been exposed to EGF for 180 min, as was the case in the absence of added growth factors, little or no *c-fos* ex-

Figure 2. Morphologic and antigenic properties of neurons derived from EGF-generated spheres of undifferentiated cells. Dual label indirect immunocytochemistry of cells within spheres cultured in the presence of 10 ng/ml BDNF for 10 DIV. Cells with neuronal morphology exhibited relatively small, round or pyramidal cell bodies with long, thin processes extending out. These cells exhibit dual immunoreactivity for NSE (A) and MAP-2 (B), as well as β -tubulin (C) and GABA (D). Scale bar, 30 μ m.

Figure 3. Cellular targets for BDNF and EGF in 3 DIV spheres of cells. EGF-generated spheres, that were rinsed free of EGF and cultured in the absence of additional growth factors for 3 DIV, were exposed to BDNF or EGF for 60 min. Spheres were immediately fixed and subsequently processed for dual label immunocytochemistry for *c-fos* (A, C) and the neuronal antigen β -tubulin (B, D). When spheres were exposed to BDNF, *c-fos* immunoreactivity (A, arrows) was restricted to β -tubulin-immunoreactive (B, arrows) cells. Under the same experimental conditions, when spheres were exposed to EGF, *c-fos* immunoreactivity (C) was found in both non-neuronal (short, open arrows) and β -tubulin-immunoreactive (D, arrow) cells. Scale bar, 30 μ m.

pression was observed. We then examined and compared *c-fos* expression in 3 DIV spheres exposed to BDNF (100 ng/ml; Collazo et al., 1992) or EGF (20 ng/ml) for 60 min (Fig. 3). We performed dual label immunocytochemistry with *c-fos* antiserum and β -tubulin antibody, the latter chosen for its characteristic as an early neuronal marker (Lee et al., 1990). In spheres exposed to BDNF, *c-fos* expression was restricted to a subpopulation of cells (Fig. 3A) that colocalized β -tubulin (Fig. 3B) and exhibited an immature neuronal morphology. These findings were observed in three independent culture preparations. In contrast, spheres exposed to EGF showed *c-fos* expression in many cells (Fig. 3C), a small proportion of which colocalized β -tubulin (Fig. 3D). Many of the cells that expressed *c-fos* in response to EGF were astrocytes (data not shown), while other responding cells expressed no antigens characteristic of differentiated cells. These results suggested that BDNF actions were restricted to and directly on neuronal cells within the EGF-generated spheres.

BDNF increases the numbers of neurons and promotes process growth

EGF-generated spheres were rinsed free of EGF and cultured on glass coverslips in media containing 1% FBS in the absence or

presence of BDNF (10 ng/ml). We previously determined that the inclusion of 1% FBS was necessary to ensure viability of EGF-generated precursor cells following removal of EGF (data not shown). The concentration of BDNF used has been demonstrated to enhance the *in vitro* survival of retinal ganglion cells (Rodriguez-Tebar et al., 1989) and septal cholinergic neurons (Alderson et al., 1990), and to enhance both the survival and neurite outgrowth of cultured cerebellar granule cells (Lindholm et al., 1993). Within 7 DIV, a difference was observed between BDNF-treated versus control spheres. Cells with neuronal morphology appeared in greater numbers, had migrated from the core of the spheres and exhibited extensive branching. This phenomenon was fully developed after 10 DIV and could be seen after fixing the spheres and immunostaining for GABA (Fig. 4). In control spheres, most neurons remained associated with the underlying glial bed and oriented neurites away from the core of the sphere (Fig. 4A). In spheres exposed to BDNF (Fig. 4B), many more neurons were observed, some remained within the core of the sphere while others migrated up to 100 μ m away, and all of them exhibited extensive neuritic branching. Quantitative analyses of NSE/MAP-2 double-labeled cells with neuronal morphology showed a twofold increase ($p < 0.001$) in

Figure 4. BDNF increases the numbers of neurons within EGF-generated spheres and enhances neuronal process growth. EGF-generated spheres that were rinsed free of EGF and cultured in the absence (A) or presence (B) of 10 ng/ml of BDNF for 10 DIV, were fixed and processed for indirect immunocytochemistry with antiserum to GABA. In the presence of BDNF, an apparent increase in neurons, all of which extended processes with extensive branching, was observed. Scale bar, 50 μ m. Quantitative analyses (C) showed that BDNF-treated spheres had twice as many neurons, relative to control. Data represent the mean $\pm$ SEM for 125 and 113 spheres in control and BDNF conditions, respectively, examined in at least four independent culture preparations; **, $p < 0.001$.

Figure 5. BDNF does not induce the proliferation of EGF-generated neuronal precursors. EGF-generated spheres were rinsed free of EGF and cultured in the presence of 10 ng/ml of BDNF and 1 μ M BrdU. Spheres were then fixed and processed for dual label indirect immunocytochemistry for neuronal (A) and glial (B) antigens and BrdU (C, D). Cells with neuronal morphology and immunoreactivity for NSE (A, small, long arrows) did not incorporate BrdU (C). On the other hand, cells expressing GFAP-immunoreactivity (B, open arrow) and those that were unreactive (B, large arrowhead) incorporated BrdU (D). Similar results were obtained with spheres that were not treated with BDNF (not shown). Scale bar, 30 μ m.

neuronal numbers in BDNF-treated spheres, after 10 DIV (Fig. 4C). Indirect immunocytochemistry for GFAP or O4 showed no significant changes in numbers of astrocytes or oligodendrocytes, respectively (data not shown).

BDNF increases in neuronal number are not due to proliferation and do not persist over longer culture periods

In order to determine whether the increase in neuronal number in spheres exposed to BDNF was due to division of neuronal precursors, spheres were rinsed free of EGF and cultured in media with 1% FBS and 1 μ M bromodeoxyuridine (BrdU), in the absence or presence of BDNF. BrdU incorporates into the DNA of dividing cells and has been used by our laboratory as a reliable assay for newly generated neurons (Reynolds and Weiss, 1992; Reynolds et al., 1992; Vescovi et al., 1993). At 10 DIV, dual label indirect immunocytochemistry with antibodies directed against NSE and BrdU revealed that virtually all NSE-immunoreactive cells, arising from either control or BDNF-treated spheres, were not BrdU-immunoreactive (Fig. 5A,C). These results were consistent in three independent culture preparations. This finding suggests that the increase in the numbers of neurons observed in the presence of BDNF was not due to proliferation of neuronal precursors. Although neuronal precursors were not dividing, BrdU incorporated into non-neuronal cells. BrdU incorporation was observed principally in flat cells, of which some

were GFAP-immunoreactive, in both control and in BDNF-treated spheres (Fig. 5B,D).

Given that there was a significant increase in the number of neurons in spheres cultured for 10 DIV in the presence of BDNF, and that this increase in neuronal numbers was not due to the proliferation of neural precursors, we next examined the time course of BDNF actions. Preliminary experiments revealed that removal of BDNF 24 hr after plating resulted in spheres that were not different than control spheres when examined at 10 DIV. These early studies also indicated that BDNF was required for at least 5 DIV or more to yield the significant increase in the numbers of neurons that morphologically and antigenically reached maturation by 10 DIV. To examine the effect of BDNF over a time period extending beyond 5–10 DIV, EGF-generated spheres were rinsed free of EGF as described above. Four to five spheres of similar size were plated by micromanipulation, onto polyornithine coated glass coverslips in each of the 24 wells of the culture plate (in 1 ml of media containing 1% FBS). BDNF (10 ng/ml) was added to 12 of the 24 wells only at plating. At 5, 10, 21, and 27 DIV coverslips were transferred from the culture plate, to another plate containing 4% paraformaldehyde for fixation and subsequent indirect immunocytochemistry for neuronal antigens. As shown in Figure 6, at 5 DIV there was no significant difference between the numbers of neurons in control versus the BDNF-treated spheres. At 10 DIV,

Figure 6. BDNF increases in neuronal numbers diminishes over time. EGF-generated spheres were rinsed free of EGF and cultured in the absence or presence of 10 ng/ml of BDNF. After the indicated days *in vitro*, spheres were fixed and the numbers of neurons counted. Significant increases in neuronal numbers were observed at 10 and 21 d *in vitro*, but not at 5 and 27 d *in vitro*. Data represent the mean $\pm$ SEM for 100–200 spheres in control and BDNF conditions, examined in at least four independent culture preparations; **, $p < 0.001$; *, $p < 0.05$.

BDNF-treated spheres contained close to twice as many neurons compared to control ($p < 0.001$). Over time the difference between the numbers of neurons generated from the spheres in the absence or presence of BDNF became less significant. Only 55% more neurons were observed in BDNF treated spheres at 21 DIV ($p < 0.05$). By 27 DIV a further decline in neuronal number was observed in BDNF-treated spheres whereas the numbers of neurons counted in control spheres did not change from those at 21 DIV. The difference between control and BDNF-treated spheres was no longer significant. These findings demonstrate that the BDNF-induced increase in neuronal numbers does not persist over time and that over time a decline in neuronal numbers occurs in spheres cultured with or without BDNF.

Quantitative analyses of neuron and neurite numbers in varying BDNF administration procedures favor a role for BDNF as a differentiation factor

If BDNF is a survival factor the numbers of neurons observed at 10 DIV should be maintained over time. Moreover, delayed administration of BDNF should result in little or no increased numbers of neurons, when compared to control. The decrease in neuronal numbers by 27 DIV suggests that BDNF is not a survival factor, rather it may enhance neuronal differentiation. Alternatively, BDNF could be a survival factor and the decrease in neuronal numbers over time could be the result of (1) depletion of BDNF in culture media, or (2) additional survival requirement for mature versus immature neurons. To distinguish between the actions of BDNF in promoting survival versus differentiation, a series of experiments were designed in which the numbers of neurons and neurites were quantified in procedures which varied with respect to administration of BDNF. EGF-generated spheres were rinsed free of EGF as described above and four to five spheres of similar size were plated by micro-manipulation directly into polyornithine-coated plastic wells of the culture plate in 1 ml of media containing 1% FBS (in these experiments glass coverslips were not used). For each experiment, two culture plates were prepared for quantification of neuronal numbers at 10 and 21 DIV. Process outgrowth was quantified at

21 DIV only. The different procedures with respect to BDNF administration consisted of (1) control, which did not receive BDNF; (2) one-time addition of BDNF at the beginning of the culture for the duration of 21 DIV; (3) exposure to BDNF for 48 hrs only, followed by its removal for the subsequent 19 DIV; (4) control for the media change, but which did not receive BDNF; (5) supplementation of BDNF, by adding 5 ng/ml every second day, throughout 21 DIV; (6) single readdition of BDNF at 10 DIV; and (7) delayed addition of BDNF at only 10 DIV. After 10 and 21 DIV these spheres were processed for immunocytochemistry using antiserum to NSE and a secondary antibody conjugated to alkaline phosphatase (developed with NBT and BCIP). Neurons and their processes were quantified in spheres from five independent culture experiments such that the reader was blind to the experimental procedures. The data obtained (Table 1) was statistically analyzed by χ^2 analysis.

Neuron numbers

In the first procedure (1 in Table 1) spheres were grown in culture media plus 1% FBS without BDNF, while spheres cultured in the second procedure (2) received 10 ng/ml BDNF once only at the time of plating. As with the time course study above, consistency in neuronal numbers was observed at two levels throughout these experiments: one corresponding to time, and the other to treatment with BDNF. As illustrated in Table 1, there were consistently more neurons per sphere at 10 DIV, in both control as well as in the presence of BDNF, than there were at 21 DIV ($p < 0.01$). However, there were consistently more neurons counted in BDNF [72% more at 10 DIV ($p < 0.001$) and 59% more at 21 DIV ($p < 0.01$)] relative to control spheres at both time intervals. At 21 DIV, neuronal numbers in control spheres had decreased by 23% in comparison to a 29% decrease in the presence of BDNF. Again, these results indicated that over an extended culture period loss of neuronal cells occurred both in the absence and presence of BDNF.

Is continuous BDNF required for increases in neuronal numbers? To determine if the continued presence of BDNF was a requirement for observing the significant increase in the numbers

Table 1. Effects of various BDNF administration procedures on neuron and neurite numbers

Procedure	Assay			Number of neurites (mean ± SEM) 21 DIV
	Number of neurons (mean ± SEM)		% change (10 → 21 DIV)	
	10 DIV	21 DIV		
1. Control	19.9 ± 1.5 (100) <i>n</i> = 93	15.3 ± 1.3 (100) <i>n</i> = 64	−23	64.6 ± 9.9 (100) <i>n</i> = 5
2. BDNF (10 ng/ml) at plating	34.3 ± 2.8 (172) <i>n</i> = 86	24.4 ± 2.1 (159) <i>n</i> = 53	−29	239.2 ± 28.2 (370) <i>n</i> = 5
3. (a) BDNF (10 ng/ml) at plating (b) Change to control media at 48 hr	22.7 ± 1.9 (114) <i>n</i> = 90	19.4 ± 1.5 (125) <i>n</i> = 92	−15	58.0 ± 8.5 (<i><</i> 100) <i>n</i> = 5
4. (a) Control (b) Change media at 48 hr	20.3 ± 2.4 (101) <i>n</i> = 79	15.7 ± 1.5 (103) <i>n</i> = 108	−23	—
5. (a) BDNF (10 ng/ml) at plating (b) BDNF (5 ng/ml) every 48 hr	33.3 ± 2.7 (167) <i>n</i> = 83	21.7 ± 2.0 (142) <i>n</i> = 121	−35	334.6 ± 48.6 (520) <i>n</i> = 5
6. (a) BDNF (10 ng/ml) at plating (b) BDNF (10 ng/ml) at 10 DIV	—	24.0 ± 2.0 (157) <i>n</i> = 97	—	215.0 ± 33.2 (333) <i>n</i> = 5
7. (a) Control — first 10 DIV (b) BDNF (10 ng/ml) at 10 DIV	—	23.7 ± 2.1 (155) <i>n</i> = 103	—	166.2 ± 22.0 (257) <i>n</i> = 5

Quantification of neuron and neurite numbers was performed as described in Materials and Methods. The values in parentheses are the calculated percentages relative to control, which was 100%. The *n* values are the numbers of single spheres analyzed, within a minimum of five independent culture preparations. Statistical comparisons are described in Materials and Methods and Results.

of neurons at 10 DIV, BDNF(10 ng/ml) was added to the culture at the time of plating and removed 48 hr later (procedure 3). BDNF was removed by aspirating the BDNF-containing media and quickly replacing it with control media. To exclude the possibility that the media change itself was responsible for any differences in neuronal numbers a sister control culture (4) was run for this procedure, in which the media was also removed at 48 hr. When spheres were exposed to BDNF for only 48 hr, the number of neurons counted at 10 DIV, was not significantly different than that counted in sister control spheres. In comparison, when spheres were allowed to remain in the presence of BDNF, 72% more neurons were counted with respect to control spheres at 10 DIV ($p < 0.001$). At 21 DIV, however, there were 25% more neurons in spheres that received the 48 hr exposure to BDNF than there were in the control spheres ($p < 0.05$), versus 59% more neurons in spheres cultured in the presence of BDNF ($p < 0.01$). It is worth noting that in control spheres run to parallel the removal procedure for BDNF, the number of neurons produced from spheres did not differ from that observed in the control procedure where there was no media change.

Is the decrease in BDNF enhanced neuronal numbers over time due to depletion of the factor? In the next two procedures, the possible depletion of BDNF in the culture media, after its addition at the time of plating, was addressed by supplementing BDNF (5 ng/ml) every 48 hr (procedure 5) or the readdition of BDNF (10 ng/ml) at 10 DIV (procedure 6). When neurons were quantified at 10 DIV there was no significant difference between

spheres that received BDNF only at the time of plating and those that received BDNF every 48 hr. Similarly at 21 DIV, there was no significant difference between neuronal numbers quantified in spheres receiving BDNF only at the time of plating and those that received BDNF continuously or ones in which BDNF was re-added at 10 DIV. Hence a continuous supply of BDNF does not further increase the numbers of neurons produced at 10 DIV nor does it prevent the subsequent loss of neurons over time.

Does delayed BDNF administration have an effect on neuronal numbers? In the last procedure (7) spheres were plated in culture wells in media plus 1% FBS only. At 10 DIV these spheres received their first exposure to BDNF (10 ng/ml) and were left undisturbed for another 11 DIV. The following observations were made at 21 DIV: (1) the numbers of neurons was not significantly different from those in spheres where BDNF was added only at the time of plating, or those in which BDNF was supplemented once (10 DIV) or every 48 hrs; (2) the number of neurons produced from spheres at 21 DIV, even after delayed addition of BDNF, was significantly greater (55%, $p < 0.01$) than neuronal counts obtained from control cultures which never received BDNF and analyzed at 21 DIV; and (3) delayed administration of BDNF at 10 DIV (and counts at 21 DIV) showed a small, but significant ($p < 0.05$) increase over the numbers of neurons in control cultures at 10 DIV.

These findings indicate that BDNF is continuously required for an action on neuron numbers and that the erosion of BDNF increases over time is not due to depletion of the factor. Delayed

administration of BDNF is capable of mimicking the increase in numbers due to BDNF being present at plating and throughout the culture period.

Neurite numbers

In the absence of BDNF (procedure 1), neurons extended processes that possessed a relatively simple outgrowth pattern. An average of 64.6 ± 9.9 neurites were counted in these spheres (see Materials and Methods for details of the counting procedure). In the presence of BDNF (procedure 2), however, long processes extended out of neuronal cell bodies in an intricate and dense network of branches. Process outgrowth in these spheres relative to control was increased by 270% ($p < 0.001$): This is clearly greater than that expected as a result of the increase in neuronal numbers which was 72%. When BDNF was removed after 48 hr (procedure 4), there was no significant difference in the numbers of neurites counted in this procedure when compared to control spheres. The greatest number of neurites (420% increase over control) was observed in spheres that were supplemented with BDNF every 48 hr (procedure 5). When BDNF was readdd (at 10 DIV) to spheres that had received BDNF at the time of plating, the numbers of neurites/sphere were not significantly different than that observed when BDNF was added at plating. Although fewer neurites were observed in spheres that received a delayed addition of BDNF (procedure 7), it was significantly more (157% increase; $p < 0.01$) than that observed in control spheres.

To determine if a continuous supply of BDNF further enhanced process growth, data from procedure 5 was compared to that obtained in procedure 2. When neurons arising from the spheres received BDNF (5 ng/ml) every 48 hr there was a 40% increase ($p < 0.01$) in process outgrowth relative to that observed in spheres receiving BDNF only at the time of plating. However when spheres plated in BDNF receive additional BDNF (10 ng/ml) at 10 DIV no significant difference in neurite outgrowth is observed over those that receive BDNF only at the time of plating. Of note here is that whereas supplementing BDNF continuously (procedure 5) does not produce an increase in the numbers of neurons when compared to those that receive a one-time supplement (procedure 6) delayed addition of BDNF (procedure 7), continuous BDNF does induce a significant increase in neuronal process growth. Although neurite outgrowth was quantified only at 21 DIV, in spheres that received BDNF at the time of plating only, a similar density of processes was observed at 10 DIV as well, while in control spheres process outgrowth was minimal (illustrated qualitatively in Fig. 3A,B).

These results demonstrate that the presence of BDNF promotes neuronal process growth and this action appears to be further enhanced by maintaining a continuous supply of BDNF in the media.

BDNF actions do not involve the enhanced differentiation of a novel neuronal phenotype

We have previously reported that when EGF-generated spheres are allowed to differentiate, the principal neurotransmitter phe-

notypes of newly generated neurons are GABA and substance P (SP) (Reynolds et al., 1992). As outlined in the introduction, examination of BDNF actions on cultures of postmitotic neurons has provided evidence for enhancement of expression of subpopulations of neuronal phenotypes. To determine if the BDNF-induced increase in neuronal numbers was associated with the induction of a novel neurotransmitter phenotype, spheres were rinsed free of EGF and plated in the absence or presence of BDNF (10 ng/ml). At 10 DIV, the spheres were fixed and processed for indirect dual label immunocytochemistry with monoclonal antibody directed towards either of the neuronal antigens β -tubulin or MAP-2 and antisera raised against a variety of neurotransmitters or synthesizing enzymes. The antisera for neurotransmitters or synthesizing enzymes tested included GABA, SP, somatostatin, neuropeptide Y, ChAT, tyrosine hydroxylase, 5-HT, met-enkephalin, and glutamate. All of these antisera have been used successfully by our lab to identify neuronal populations in primary embryonic mouse CNS cultures. The presence of BDNF did not induce the expression of any new neurotransmitter phenotypes in neuronal cells within the spheres. Cells with neuronal morphology, colocalized GABA (Fig. 7A) and SP (Fig. 7B). In fact, 96% of all neurons immunoreactive for GABA ($n = 316$ neurons) in BDNF-treated cultures, and 87% of all GABAergic cells in control conditions ($n = 83$ neurons) also exhibited immunoreactivity to SP. The restriction of BDNF actions to this one phenotype was further substantiated by independent quantification of GABA and SP neuronal numbers (Fig. 7C). In the presence of BDNF there were more GABA⁺ and more SP⁺ neurons than there were in control cultures ($p < 0.001$). The results also indicated no significant difference in the numbers of GABA⁺ and SP⁺ neurons within either the BDNF-treated cultures or the untreated cultures, confirming that the two neurotransmitters were colocalized. From these data, we conclude that the effects of BDNF are not inductive or selective for a particular neurotransmitter phenotype, rather BDNF acts equally upon a single population of EGF-generated neuronal precursors.

Discussion

The expression of *trkB* in newly formed structures of the mammalian CNS, as well as in the adult (Klein et al., 1990), supports a role for the principal ligand BDNF in neural development. Of the three neurotrophin receptors examined in this study, only *trkB* was expressed in appreciable quantities in spheres of undifferentiated cells generated by EGF-responsive stem cells. BDNF directly enhanced the differentiation of neuronal precursors within the spheres, this action being characterized by a transient increase in neuronal numbers and a pronounced action on neurite outgrowth. BDNF did not, however, enhance the long term survival of these same neuronal precursors, regardless of the manner of administration. It is plausible to suggest that BDNF may act subsequent to mitogens such as EGF (Reynolds et al., 1992) and bFGF (Vescovi et al., 1993) in the restriction/differentiation of newly generated neurons derived from stem

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Figure 7. BDNF actions are on a single population of neurons that colocalize GABA and substance P. EGF-generated spheres that were rinsed free of EGF and cultured in the absence or presence of 10 ng/ml of BDNF for 10 DIV, were fixed and processed for dual label indirect immunocytochemistry with antibody to GABA (A) and antiserum to substance P (B). Ninety-six percent of GABA-immunoreactive neurons were also immunoreactive for substance P ($n = 316$ neurons). Scale bar, 30 μ m. Quantitative analyses (C) indicated that BDNF treatment resulted in similar, significant augmentation in the numbers of neurons expressing both GABA and substance P. Data represent the mean $\pm$ SEM for 38–52 spheres in control and BDNF conditions, examined in at least four independent culture preparations; **, $p < 0.001$.

cell precursors. Additional factors are likely required for long term neuronal survival.

In studies of the PNS, neurotrophins have been demonstrated to play roles in the proliferation, differentiation and survival of neuronal precursors (reviewed in Davies, 1994). In contrast, the vast majority of studies of neurotrophin actions in the CNS have focused on postmitotic neuronal populations. Given the expression of *trkB* in the neuroepithelium as early as E9.5 in the rat (Klein et al., 1990), perhaps it is not surprising that this receptor is expressed on undifferentiated cells derived from an embryonic neural stem cell. Both the catalytic domain-containing and non-catalytic (truncated) forms of *trkB* are expressed in the stem cell progeny, however *c-fos* immunoreactivity studies suggest that the former is restricted to neuronal cells. The location and function of the truncated form in the stem cell progeny remains undetermined. Despite the presence of *trkB* on the stem cell-derived mitotically active cells, BrdU incorporation studies suggest that the actions of BDNF do not include the stimulation of neuronal precursor cell division. Such activity is consistent with virtually all studies of neurotrophin actions on dissociated embryonic CNS tissue. NGF has been reported to enhance striatal neuron precursor cell division, however this action required pretreatment with bFGF (Cattaneo and McKay, 1990).

After spheres of undifferentiated cells were exposed to BDNF for 10 DIV, there was a 1.7–2-fold increase in neuronal numbers. This significant increase, however, decreased over time and was not apparent after 27 DIV. This decrease was not due to depletion of BDNF in the medium and thus suggests that BDNF does not enhance long term neuronal survival nor is it capable of preventing a decline in neuronal numbers. In fact, delayed exposure to BDNF was as effective as BDNF administration throughout the culture period in increasing neuronal numbers. Moreover, the delayed administration of BDNF at 10 DIV actually yielded more neurons (although this just achieved statistical significance at 21 DIV) than were present prior to exposure to the factor. In addition, a 48 hr exposure to BDNF is sufficient to induce an increase in neuronal numbers after 21 DIV, although not as great as that due to continuous administration. We interpret these results as indicative of an action of BDNF in either enhancing the antigenic differentiation of committed immature neurons or directing the fate of uncommitted precursors with neuronal potential (discussed further below). Although some earlier studies of dissociated embryonic CNS cells concluded that BDNF enhanced neuronal survival (Alderson et al., 1990; Hyman et al., 1991, 1994), some of those conclusions may be reconsidered in light of several issues. More than an issue of semantics and taking into account observations made in our study, the data in those studies could be interpreted as enhancement of neuronal differentiation rather than survival. In those studies, the focus was on septal cholinergic (Alderson et al., 1990) and mesencephalic dopaminergic neurons (Hyman et al., 1991), each of which comprises 1–5% of the total neuronal population examined. While the total neuronal numbers did not change, BDNF did enhance the numbers of cholinergic (AChE-immunoreactive) and dopaminergic (tyrosine hydroxylase-immunoreactive) neurons by two- to threefold. The authors showed that delayed addition of BDNF resulted in a reduction in neuron numbers and thus argued for a survival role. However, in neither case was the delayed exposure carried out for the same period of time as those exposed without delay. Moreover, the authors did not examine extended periods in culture to determine whether BDNF could enhance these phenotypes over the long term.

In any case, the findings of those studies could have been interpreted as BDNF-induced enhancement or switch of neuronal phenotype, which may only be possible over a narrow window of time.

Given the results of many other studies (including those examining similar neuronal populations) which clearly demonstrate that BDNF is a differentiation factor, that is, enhancing the neurotransmitter phenotype properties of neurons without affecting neuronal numbers (Knusel et al., 1991; Ip et al., 1993; Mizuno et al., 1994; Nawa et al., 1994), there is little evidence for BDNF as a long term survival factor for CNS neurons. Interestingly, studies of retinal ganglion cells (Johnson et al., 1986; Rodriguez-Tebar et al., 1989) and cerebellar granule neurons (Segal et al., 1992; Lindholm et al., 1993) demonstrate an increase in total cell number in response to BDNF, when measured after 4–7 DIV. Again, while the authors argue that this is indicative of an action on neuronal survival, in both systems long term survival was not measured. Moreover, in both systems the role of BDNF in enhancing neuronal numbers is transient (Rodriguez-Tebar et al., 1989; Segal et al., 1992), whereby the same populations derived from older animals are no longer susceptible to BDNF (actions on process growth in older preparations was not examined). Nevertheless, these findings support a role for BDNF as a commitment/short-term survival factor, whose long term survival is dependent on additional factors and/or other neurotrophins.

In addition to its ability to enhance neurotransmitter phenotype, some of the studies of BDNF actions on CNS neuron populations have included observations of enhanced neurite outgrowth (Alderson et al., 1990; Mizuno et al., 1994). In the present study, we observed a BDNF enhancement of neurite outgrowth that was entirely dependent on the continued presence/addition of the factor. Several conclusions arise from our quantitative analyses. First, although a 48 hr exposure to BDNF is not sufficient to enhance neuritic growth, administration of the factor at plating is more effective than delayed administration in inducing neuritic outgrowth, suggesting that younger cells are more susceptible to the neurite enhancing effect. Second, repeated addition (every 2 d), but not a one time addition at 10 DIV, of BDNF resulted in a significant increase in neurites without any increase in neuronal numbers. The final and perhaps most significant conclusion is that all the actions of BDNF on neurites are independent of neuronal numbers. Interestingly, in the only other quantitative assessment of these phenomena (Lindholm et al., 1993), cerebellar granule cells also exhibited robust neuritic growth independent of neuronal numbers.

An observation made in this study, and apparent in reviewing the literature, is that some neurons will exhibit differentiation/short-term survival in the absence of BDNF. Although rarely addressed, this observation may serve to further elucidate the precise role that BDNF plays in neuronal development. We propose two explanations for this phenomenon. The first is that there are two populations of neuronal cells, derived from the EGF-responsive stem cell—one that is BDNF-responsive, the second nonresponsive. In fact, we are aware of at least two types of neuronal precursors in the spheres of undifferentiated cells, one a unipotent neuroblast and the other a bipotent neuronal/astroglial precursor (Vescovi et al., 1993). However, several observations argue against differential susceptibility of neuronal subpopulations. Only one neuronal phenotype, a GABA-SP-colocalizing neuron, emerges consistently from these spheres of undifferentiated cells. In contrast to other studies (Ip et al., 1993; Mizuno et al., 1994), no additional phenotypes emerge as a con-

sequence of enhanced differentiation. In addition, when assayed by *c-fos* immunoreactivity or by enhancement of neurite outgrowth, all cells with neuronal morphology and antigenicity appear to respond to BDNF. If the neurons that are present in the absence of BDNF were unresponsive, we would expect that at least half of all neuronal cells in the spheres would show no *c-fos* or process growth in response to BDNF—however, this is not the case. Thus, we propose that the actions of BDNF on cells within the spheres, with respect to increases in neuronal numbers, is a stage-dependent phenomena. Stage dependency has been proposed as an explanation for transient responsiveness, to BDNF, of cells derived from both the PNS (Buchman and Davies, 1993) and CNS (Rodriguez-Tebar et al., 1989; Knusel et al., 1991; Segal et al., 1992). Thus, once neurons have reached a critical period in their differentiation, BDNF is no longer required or ineffective in regulating their further differentiation/long term survival. In spheres of undifferentiated cells, derived from the EGF-responsive stem cell, it is conceivable that cells that are born first and/or have been exposed to cell–cell or cell–extracellular matrix interaction, commit to the neuronal lineage and undergo initial differentiation, do not require exposure to BDNF to achieve this stage. Alderson et al. (1990) demonstrated that cell density determined the extent of BDNF responsiveness, whereby the actions of BDNF in increasing neuronal numbers was only apparent at densities where cell–cell contact or paracrine growth factor release was minimized. Birthdating of cells during their genesis from the EGF-responsive stem cell could address the issue of whether first born cells are more differentiated and not requiring of BDNF.

Despite ruling out a long term survival role and demonstrating a differentiation role with respect to neurite outgrowth, this study leaves one issue unresolved: what underlies the BDNF-induced increase in neuronal numbers that becomes particularly apparent between 5 and 10 DIV? Our results with BrdU incorporation suggest that this action is not due to the proliferation of neuronal precursors. BDNF may simply act to enhance the expression of antigens characteristic of mature neurons. This may be addressed by examining the expression of proteins, that is, voltage-gated calcium channels, synaptic vesicle proteins, etc., after short term administration of BDNF to young (5–7 DIV) sphere cultures. Increases in some of these proteins prior to the increase in neuronal numbers would confirm such a hypothesis. An alternative explanation might be that BDNF influences the fate of uncommitted precursors, or less likely the trans-differentiation of committed precursors, towards the neuronal lineage. Single cell clonal studies, of undifferentiated cells cultured first in the absence or presence of BDNF with subsequent removal of the factor and the addition of a differentiation medium, would address whether BDNF induces/restricts the fate of uncommitted CNS precursor cells. Such approaches were used to demonstrate that BDNF could direct pluripotent neural crest cells towards the primary sensory neuron lineage (Sieber-Blum, 1991).

In this study, the actions of BDNF on process outgrowth of stem cell-derived neuronal cells persists, although BDNF does not prevent the long term decline in neuronal numbers. Other factors, acting in concert with or in place of BDNF, are presumably required for long term neuronal survival. Tetrodotoxin-sensitive electrical activity has been demonstrated to play an important role in the long term survival of cultured CNS neurons (Lipton, 1986; reviewed in Johnson and Deckworth, 1993). Ghosh and colleagues (1994) found that depolarization acts in conjunction with an autocrine/paracrine action of BDNF to en-

hance the survival of cultured cortical neurons. Examination of the dependency of cerebellar granule cells found that BDNF actions in neuronal commitment are followed by a role for NT-3, acting through the *trkC* receptor, in further, long term neuronal differentiation/survival (Segal et al., 1992). We are currently examining the role that BDNF may play in inducing the expression of other *trk* receptors on the stem cell neuronal progeny. Another phenomenon that may participate in the further differentiation of *trkB* expressing neuronal cells could be the actions of the other principal agonist of this receptor—NT-4/5. Hyman and co-workers (1994) reported the surprising finding that some of the actions of NT-4/5, presumably through the same receptor, may be distinct from those of BDNF. Some of these processes may contribute to the final stages of differentiation/long term survival of neurons derived from the EGF-responsive stem cell.

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