Stage-specific and opposing roles of BDNF, NT-3 and bFGF in differentiation of purified callosal projection neurons toward cellular repair of complex circuitry

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Keywords: growth factors, mouse, neocortex, neurotrophins, regeneration

Abstract
Cellular repair of neuronal circuitry affected by neurodegenerative disease or injury may be approached in the adult neocortex via transplantation of neural precursors ("neural stem cells") or via molecular manipulation and recruitment of new neurons from endogenous precursors in situ. A major challenge for future approaches to neuronal replacement will be to specifically direct and control progressive differentiation, axonal projection and connectivity of neural precursors along a specific neuronal lineage. This goal will require a progressively more detailed understanding of the molecular controls over morphologic differentiation of specific neuronal lineages, including neurite outgrowth and elongation, in order to accurately permit and direct proper neuronal integration and connectivity. Here, we investigate controls over the morphologic differentiation of a specific prototypical lineage of cortical neurons: callosal projection neurons (CPN). We highly enriched CPN to an essentially pure population, and cultured them at three distinct stages of development from embryonic and postnatal mouse cortex by retrograde fluorescence labelling, followed by fluorescence-activated cell sorting. We find that specific peptide growth factors exert direct stage-specific positive and negative effects over the morphologic differentiation and process outgrowth of CPN. These effects are distinct from the effects of these growth factors on CPN survival [Catapano et al. (2001) J. Neurosci., 21, 8863–8872]. These data may be critical for the future goal of directing lineage-specific neuronal differentiation of transplanted or endogenous precursors/"stem cells" toward cellular repair of complex cortical circuitry.

Introduction
Cellular repair of complex, long-distance neocortical circuitry affected by neurodegeneration or injury may be possible via transplantation of neural precursors or via induction of neurogenesis in situ and recruitment of new neurons from endogenous neural precursors, or "neural stem cells" (Magavi et al., 2000; Arvidsson et al., 2002; Nakatomi et al., 2002; Parent et al., 2002). Neuronal replacement strategies will almost certainly require an understanding of extracellular controls that guide the survival and directed differentiation of such precursors and partially differentiated and committed immature neuroblasts along desired lineages. Of particular importance to the differentiation and successful connectivity of projection neurons, these control factors must exert the appropriate balance of both stimulatory and inhibitory effects on neurite outgrowth and elongation. Using heterogeneous cortical slice and culture preparations, peptide growth factors that promote neurite outgrowth have been shown to regulate initial process outgrowth, elongation and branching (McAllister et al., 1995, 1997; Horch et al., 1999; Le Roux et al., 1999; Szelenyi et al., 2001). In similar heterogeneous preparations, inhibitory signals have been shown to control neurite number and length, and may be required to slow neurite elongation in order to allow for branching (Halloran & Kalil, 1994; Szelenyi et al., 1998, 2001). Although these and other studies have demonstrated effects on heterogeneous populations of neurons and, therefore, form the framework for understanding the range of potential growth factor effects, the lack of highly enriched or purified populations for study limits their relevance to the identification of direct, cell-autonomous controls, for which highly enriched or purified neuronal populations are required.

In the studies reported here, we investigate, for the first time, control by growth factors over the development of a specific projection neuron lineage: callosal projection neurons (CPN). CPN are prototypical cortical projection neurons that reside in layers II/III and V of neocortex and project via the corpus callosum to targets in homologous regions of contralateral cortex (Conti & Manzoni, 1994). CPN and other related long-distance projection neurons in the cortex are vulnerable in a number of neurodegenerative diseases, e.g. interhemispheric callosal neurons in Alzheimer’s disease (Pearson et al., 1985; Hampel et al., 1998), cortico-basal ganglionic neurons in cortico-basal degeneration (Yamauchi et al., 1998), cortico-striatal neurons in Huntington’s disease (Sapp et al., 1999) and cortico-spinal neurons in amyotrophic lateral sclerosis (Jackson & Bryan, 1998). Cortical CPN appear to undergo abnormal development in autism spectrum disorders (Egaas et al., 1995; Piven et al., 1997). Thus, CPN provide a prototypical population of cortical projection neurons that degenerate or are injured in a variety of human diseases.

We have previously shown that transplanted neural precursors and neuroblasts, and endogenous precursors that have been recruited by induction of adult neurogenesis, can differentiate into long-distance
projection neurons and integrate even within the highly inhibitory environment of the adult neocortex, under appropriate conditions (Macklis, 1993; Sheen & Macklis, 1995; Hermit-Grant & Macklis, 1996; Snyder et al., 1997; Leavitt et al., 1999; Sheen et al., 1999; Magavi et al., 2000; Shin et al., 2000; Fricker-Gates et al., 2002). Work in the avian songbird forebrain demonstrates that similarly recruited neurons derived from endogenous precursors can contribute to functional circuitry and restore a learned behaviour (Scharf et al., 2000). These results make clear that understanding the molecular controls over lineage-specific survival and differentiation of projection neurons will be of critical importance to direct and optimize projection neuron differentiation and survival in vivo and, thus, increase the efficiency of cellular repair by manipulation of neural precursors. Although it is not clear whether the same sequence and combination of molecules that control CPN survival and differentiation during development also control CPN maturation from transplanted or endogenous neural precursors during neuronal incorporation or induced adult neurogenesis, it is very likely that embryonic and adult neurogenesis may share at least some critical molecular controls.

Until recently, investigation of growth factors and neurotrophins that control lineage-specific survival and differentiation has been limited by the lack of lineage-specific markers that could allow isolation of pure neuronal populations of a specific lineage. To overcome these limitations, we developed methods to highly enrich CPN by fluorescence-activated cell sorting (FACS) to an essentially pure population, and culture them at several critical stages of development (Catapano et al., 2001). In those previous experiments (Catapano et al., 2001), we identified peptide growth factors that exert stage-specific survival effects on this lineage.

In the experiments presented here, we investigate the ability of selected candidate peptide growth factors to promote distinct and critical aspects of CPN morphologic differentiation and process outgrowth such as cell polarity, dendrite and axon outgrowth, process elongation and branching. We found that brain-derived neurotrophic factor (BDNF), NT-3 and basic fibroblast growth factor (bFGF) exhibit distinct, stage-specific and counteracting effects on potentially critical elements of morphologic differentiation of CPN purified from embryonic day 19 (E19), postnatal day 2–3 (P2–3) and P6–7 mouse neocortex.

We found that these effects on differentiation are independent of effects on cell survival by confirming these results with CPN from Bax−/− neurons that are protected from neuronal apoptosis following trophic factor withdrawal (Knudson et al., 1995). The experiments presented here thus provide a first step in elucidating controls over morphologic differentiation and process outgrowth of specific lineages of cortical projection neurons, with the future goal of functional cellular repair of complex, long-distance cortical circuitry.

Materials and methods

CPN labelling, dissociation and purification

CPN were enriched to approximately 99.5% purity by retrograde labelling and FACS-sorting from three specific stages of development in C57/B16k6 mice (Charles River Laboratories): (i) E19, when motor-sensory CPN axons first extend to the midline via the corpus callosum (Floeter & Jones, 1985); (ii) P2–3, when their axons begin to innervate contralateral cortex; and (iii) P6–7, when target innervation is complete and a subpopulation of cortical neurons undergo developmental cell death (Sppearlifico et al., 1995). Labelling and purification of CPN on the basis of their axonal projections to contralateral cortex produces a largely homogenous population of callosally projecting neurons. Purified CPN survive in isolated co-culture with cortical cells and acquire appropriate, cell type-specific morphology and expression of neurotransmitters and growth factor receptors. CPN in culture undergo apoptosis following withdrawal of exogenous trophic support. Specific peptide factors, including BDNF, NT-3, NT-4, insulin-like growth factor 1 and glial cell line-derived neurotrophic factor promote CPN survival in a stage-specific manner.

CPN from E19, P2–3 and P6–7 mice were purified as previously described (Catapano et al., 2001). Pups were anaesthetized using hypothermia and neocortical CPN were retrogradely labelled with green fluorescent microspheres (Lumafluor, Naples, FL) injected into target regions in contralateral cortex. The fluorescent latex microspheres are taken up by axon terminals and retrogradely transported to cell bodies, thus labelling contralaterally projecting CPN in layers II/III and V in the hemisphere opposite the injection site. For embryonic injections, E17–18 pregnant mice were deeply anaesthetized with Avertin and each embryo was injected through the uterine wall in motor-sensory cortex. Two injection sites per embryo were performed, with a total of 40–60 nl of green fluorescent microspheres per injection site. The pregnant dams were deeply anaesthetized 1 day after surgery, and the embryos were removed. For postnatal injections, mice were anaesthetized by hypothermia and received eight injections in motor-sensory cortex, with 40–60 nl per injection site. Mice were returned to the care of the mother and neurons were isolated from their labelled hemispheres 2 days after retrograde labelling.

CPN dissociation and purification

Twenty-four to 48 h after microsphere injection, hemispheres contralateral to injection were removed from each pup, and motor-sensory cortices were dissected in cold (4 °C) dissociation medium (containing glucose, 20 mM; kynurenic acid, 0.8 mM; APV, 0.05 mM; penicillin-streptomycin, 50 μg/mL and 0.05 mg/mL, respectively; Na2SO4, 0.09 mM; K2SO4, 0.03 M; MgCl2, 0.014 M). Retrogradely labelled cortices were pooled and gently enzymatically dissociated in medium containing L-cysteine HCl (0.016 g/L) and papain (11.7 μg/mL) at 37 °C for 30 min. Papain digestion was then blocked with ovomucoid (10 mg/mL) and bovine serum albumin (BSA; 10 mM/L) in dissociation medium at room temperature. Neurons were mechanically dissociated via gentle trituration in iced OptiMem containing glucose, 20 mM; kynurenic acid, 0.4 mM; APV, 0.025 mM. All chemicals are from Sigma and media from GibcoBRL unless otherwise noted. To increase cell viability the tissue was triturated at intervals of five–six passages through a 5–ml plastic pipette followed by precipitation of multicellular aggregates on ice to obtain a single-cell suspension. Microsphere-labelled CPN were purified from the cortical cell suspension by FACS using a FACS Vantage flow cytometer (Becton Dickinson), gating for both fluorescence and appropriate cell size, to eliminate cellular debris or potential multicellular aggregates (Fig. 1). These methods typically yield 200,000–300,000 purified CPN per FACS sort, approximately 3–5% of the total number of cells sorted. Approximately 50% of E19 CPN survive in culture at 2DIV, while P2–3 and P6–7 CPN display reduced levels of viability in culture, with approximately 10% surviving at 2DIV. All animal studies were performed in accordance with institutional and federal guidelines.

CPN culture

CPN were cultured as previously described (Catapano et al., 2001), with minor modifications. In brief, wild-type CPN were plated on poly-L-lysine-coated (Sigma) glass coverslips (Fisherverd Scientific Microscope Cover Glass) at approximately 4 × 103 cells/coverslip for E19 CPN and 1.5 × 103–2.5 × 103 cells/coverslip for postnatal CPN. These plating densities resulted in approximately 1 × 102 live CPN/coverslip for CPN at all stages after 3DIV in conditioned medium (CM) (see below). CPN at this concentration did not have direct

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physical contact with one another, allowing individual neuron morphologies to be unambiguously examined. CPN were plated in the following conditions: (i) in serum-free medium (SFM) as control (BSA, 0.034 g/L; L-glutamine, 1 mM; penicillin-streptomycin, 25 μg/mL and 0.025 mg/mL, respectively; glucose, 35 mM; 0.5% B27 (GibcoBRL) in Neurobasal medium (GibcoBRL); (ii) in CM (SFM conditioned overnight by cortical glia and neurons); or (iii) in SFM containing individual or multiple peptide growth factors at 25 ng/mL [BDNF (Peprotech, London, UK); NT-3 (Peprotech, London, UK); ciliary neurotrophic factor (CNTF; Alomone, Jerusalem, Israel); and/or bFGF (Sigma) with heparin, 0.1 μg/mL]. In initial experiments performed to narrow down a broad list of candidate factors to those with effects on CPN morphology, nerve growth factor (25 ng/mL; Alomone), NT-4/5 (25 ng/mL; Alomone), glial cell line-derived neurotrophic factor (25 ng/mL; Alomone), platelet-derived growth factor (PDGF) (25 ng/mL; Upstate Biotechnology, Lake Placid, NY, USA), leukemia inhibitory factor (LIF) (25 ng/mL; Alomone), insulin-like growth factor 1 (25 ng/mL; Peprotech), neuregulin (NRG) (1 nM; gift of G. Corfas), bone morphogenetic protein-4 (BMP-4) (25 ng/mL and 100 ng/mL; The Genetics Institute) and/or the cAMP elevator forskolin (Sigma, 5 μM) were added individually to SFM.

**CPN morphology assays**

At 3DIV, CPN morphology was visualized with the vital dye calcein, and dead cells were counterstained with ethidium homodimer EthD-1 (Live/Dead Viability/Cytotoxicity Kit, Molecular Probes, Eugene, OR, USA). For each experiment, 30 CPN in each condition [SFM, CM or SFM plus growth factor(s)] were randomly selected by moving the microscope stage two field-widths in the x and/or y directions, and identifying the cell closest to the cross-hairs in the centre of the field. A digital image of each cell was captured and stored for later blinded morphological analysis. Only CPN whose cell bodies and processes were not in contact with neighbouring cells were included in the analysis. Three–five separate experiments were performed for each experimental condition at each age. We measured cell soma size, axonal length, number and length of dendrites, and several components of dendritic complexity: number and length of primary and secondary branches; number of minor processes; number of filopodia; and total dendritic length for each imaged CPN, using NIH Image software version 1.62. We calculated average dendrite length as:

\[(\text{total neurite length} - \text{length of axon})/(\text{total number of neurites} + \text{number of branches} - 1)\]

The number of secondary branches was typically <1 for each cell, so these data are not presented independently. Only cellular processes >10 μm were considered neurites for these analyses (Dotti et al., 1988; Ziv & Smith, 1996). Processes <10 μm emanating from the cell body were defined as minor processes (Dotti et al., 1988), and processes <10 μm on the shafts or tips of dendrites, axons or growth cones were considered filopodia. Camera lucida drawings were derived from Photoshop images for Fig 3. The diameters of all processes were drawn with uniform width.

For initial experiments to select candidate factors for more detailed analysis, CPN morphology was quantified by Sholl analysis (Sholl, 1953). Seven samples of 20–30 viable CPN each (approximately 150–200 CPN per candidate factor) were analysed morphologically for each candidate factor, with very reproducible results. Briefly, concentric circles were superimposed on images of CPN, marking radii from the surface of the cell soma in 10-μm increments. For each circle, the number of intersections with neurites was counted.

Analysis of CPN morphology was performed in a blinded manner. For a subset of experiments, analysis was performed by two independent blinded observers to ensure reproducibility. Error bars represent

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**Fig. 1.** CPN purification by retrograde labelling and fluorescence-activated cell sorting (FACS). (A) Low-magnification fluorescence photomicrograph showing CPN in E19 neocortex labelled with fluorescent microspheres at E18. (B) Low-magnification fluorescence photomicrograph showing CPN in P6 neocortex labelled with fluorescent microspheres at P4. II/III–VI; cortical laminae II/III–VI; ez, ependymal zone; pia, pial surface. Similar results are obtained when labelling P2–3 CPN (Catapano et al., 2001; data not shown). (C) Sample FACS plot of the population of neurons selected. The box within the plot indicates the subpopulation (3–4%) of highly labelled CPN that are isolated. (D–G) FACS purification results in a homogeneous neuronal population of labelled CPN. Only a few percent of dissociated cortical cells before FACS purification (D) are labelled (E), while essentially all FACS-purified CPN (F) are labelled (G).
standard error of the mean. For statistical analysis, values for CPN in each experimental condition were compared with values in SFM from the same experiments. Due to the non-normal distribution of the data, statistical significance was calculated by rank sum tests. Student’s t-tests were also performed to confirm rank sum test results. T-test results closely matched those of the rank sum tests for all data. Data were normalized only for the additivity experiments and, in this case, statistics were performed on raw data before normalization.

**BrdU labelling**

Dividing cells were labelled by BrdU administration into culture medium (20 µM) at 1, 2, and 3DIV. Cells were fixed at 3–4DIV and BrdU uptake was assessed by immunocytochemistry (see below). Cytoglial cultures were labelled with BrdU in parallel as a positive control.

**Immunocytochemistry**

To distinguish CPN dendrites from axons, CPN were labelled with the somato-dendritic marker Map-2 (2a + 2b, 1: 500; or clone HM-2, 1: 500; Sigma) or axon-specific neurofilament heavy chain, NF 200 kDa (monoclonal, 1: 50; polyclonal, 1: 400; Sigma). CPN cultures at 3DIV were fixed with cold 4% paraformaldehyde in phosphate-buffered saline (PBS), washed, blocked in 5% BSA, 3% goat serum and 0.1% Tween-20, incubated overnight in primary antibody at 4°C, washed, incubated in secondary antibody antimouse Alexa 546 (1: 500) or antirabbit Alexa 488 (1: 250) for 2 h at room temperature, washed and mounted in PBS. For BrdU immunocytochemistry, CPN and control cultures were fixed with cold 4% paraformaldehyde in PBS, washed, incubated in 2 M HCl for 1 h, washed, blocked in 5% BSA, 3% goat serum and 0.1% Triton-X or 0.1% Tween-20, incubated overnight in rat anti-BrdU (1: 400; Accurate Antibodies, Westbury, NY, USA), washed, incubated in secondary antibody antimouse Alexa 546 (1: 500) for 2 h, washed and mounted in Fluoromount (VWR). Appropriate positive and negative controls, including omission of the primary antibodies, were performed in all experiments to ensure specificity of staining.

**Bax<sup>−/−</sup> animals**

A Bax heterozygote (Bax<sup>+/−</sup>) breeding pair was obtained from Jackson Laboratories (Bar Harbor, ME, USA). Bax<sup>+/−</sup> breeders were housed (as per IACUC standards) in a 12h light : dark room and given food and water ad libitum. P1–2 Bax<sup>−/−</sup> pups were identified from the litters following polymerase chain reaction (PCR)-based genotyping (see below) and injected with fluorescent latex microspheres. Contra lateral motor-sensory cortex was dissociated 1–2 days after injection for CPN purification, as described for wild-type mice.

**PCR-based genotyping**

To identify the genotype of mouse pups from Bax<sup>+/−</sup> × Bax<sup>−/−</sup> matings, tail samples were individually digested in 0.5 mL of lysis buffer (in mM: Tris-HCl, 100, pH 8.5; EDTA, 5, pH 8.0; 0.2% sodium dodecyl sulphate; NaCl, 200) with protease K (400 µg/mL) for 3–12 h at 60°C with agitation. Genomic DNA was extracted with phenol and chloroform, precipitated with isopropanol, and resuspended in Tris, 10 mM EDTA 1 mM (TE) (pH 7.5–8.0) to a final concentration of 100 ng/µL. Two separate PCR reactions were performed with 2 µL of each genomic DNA sample using primers recognizing the intact Bax sequence [wild type (WT) reaction] or the neocasette used to replace the Bax sequence [knock out (KO) reaction]. The WT reaction included Bax forward primer (5′-GAGCTGACAGAACATCACTG-3′) and Bax reverse primer (5′-GTGGTACAGATGGCGT-3′). The KO reaction included neoreverse primer (5′-CCGTTCTATATGCTCACGGG-3′) instead of the Bax reverse primer. All reagents used for PCR were from Sigma. Samples were separated on a 1% agarose gel with appropriate DNA size markers. As expected, PCR genotyping yielded a 304-bp product for the wild-type gene and a 507-bp product for the mutant gene. Litters of mice generated from Bax<sup>+/−</sup>×Bax<sup>−/−</sup>pairs resulted in the expected 1:2:1 ratios of Bax<sup>+/−</sup>, Bax<sup>−/−</sup> and Bax<sup>−/−</sup> pups, respectively. While PCR-based genotyping was consistently employed, the genotype of Bax<sup>−/−</sup> pups could also be reliably predicted by lighter coloured skin and eyes (clearly visible through the skin at P1–3).

**Bax<sup>−/−</sup> CPN survival and apoptosis assays**

Because of increased survival, postnatal Bax<sup>−/−</sup> CPN were plated at 1–2 × 10<sup>5</sup> cells/coverslip. At this concentration, Bax<sup>−/−</sup> CPN did not have direct physical contact with one another, allowing individual neuron morphologies to be unambiguously examined. Bax<sup>−/−</sup> CPN survival in SFM, CM and SFM containing individual growth factors was assessed by morphology or by staining with the vital dye calcine and exclusion of ethidiumD-1 (EthD-1; Live/Dead Viability/Cytotoxicity Kit, Molecular Probes). Apoptotic Bax<sup>−/−</sup> and wild-type CPN were visualized by condensation of nuclei labelled with Hoechst, EthD-1 or propidium iodide (0.1 mg/mL; Calbiochem, San Diego, CA, USA) at 1DIV.

**Results**

We previously reported purification (to >99.5% purity) of this distinct lineage of callosal projection neurons at the same critical stages through development as studied here (Catapano et al., 2001, Fig. 1). We found that retrogradely labelled CPN acquire appropriate pyramidal neuron morphologies in vitro, after purification by FACS, despite axotomy during dissociation and cell sorting. Specifically, purified CPN extend multiple neurites in vitro that possess varicosities, filopodia and growth cones, characteristic of developing neurons in vivo. Purified CPN express markers of neuronal differentiation and polarity, including somato-dendritic Map-2, axonal NF and the mature neuronal nuclear transcription factor NeuN (Catapano et al., 2001). This powerful model system permits analysis of the direct effects of individual growth factors/neurotrophins on morphologic differentiation in order to identify signals that may direct CPN differentiation toward neuronal replication and cellular repair in the adult neocortex.

We used somato-dendritic Map-2 (data not shown) and axon-specific NF-H to distinguish CPN dendrites from axons (Fig. 2A and B). Consistent with previous results using hippocampal neurons (Dotti et al., 1988), the longest neurite extended by purified CPN was typically Map-2-negative and NF-H-positive. This allowed us for the remainder of our analysis to identify the longest neurite as the axon. CPN axons in vitro were typically hundreds of microns long, and could extend up to >1.5 mm (Fig. 2C).

A subset of the candidate growth factors exerted direct cell-specific effects on CPN process differentiation and complexity

Following our previously reported identification of the complement of growth factor receptors expressed by FACS-purified CPN (Catapano et al., 2001), we investigated whether activating ligands for these receptors influenced CPN morphology. We initially assessed CPN process outgrowth in SFM with single peptides added: NGF; BDNF; NT-3; NT-4/5; PDGF; bFGF; CNTF and NGF. We also assessed the effects of forskolin, which elevates intracellular cAMP. At 3DIV, CPN morphology under each of these conditions was quantified by Sholl analysis (Sholl, 1953; data not shown), and compared with CPN morphology in SFM and cortical cell-CM. In this initial screen, only
BDNF, NT-3, bFGF and CNTF (Fig. 3) demonstrated significant effects on CPN morphology; the remainder did not. Our previous data confirmed that purified CPN express receptors for these growth factors: TrkB, TrkC, FGFR1 and CNTFR-alpha, respectively (Catapano et al., 2001). These four growth factors were selected for further and more detailed analysis.

**Growth factors exert distinct effects on CPN dendritic complexity vs. axonal elongation at P2–3 and P6–7**

At P2–3, CPN in SFM typically exhibited three–five primary dendrites and one–three primary dendritic branches, with an average dendrite length of 40 μm and an average axon length of 160 μm. P2–3 CPN in CM displayed substantially increased axon length, and a modest increase in dendritic complexity and soma size (Figs 4 and 5), compared with CPN in SFM. BDNF and NT-3 significantly increased dendritic complexity and soma size, but did not alter axonal or dendritic length (Figs 4 and 5). CNTF had no significant effect on dendritic number/complexity, and only minimally decreased axon length. These effects are summarized and compared with those for bFGF (below) in Table 1.

In striking contrast, bFGF significantly and specifically decreased both dendritic number/complexity and axon length, as well as uniquely reducing soma size (Figs 4 and 5). These effects of bFGF were attenuated by addition of bFGF-neutralizing antibodies (data not shown). Because heparin can potentiate the mitogenic effects of bFGF, we investigated whether heparin at a range of concentrations also potentiates these differentiative effects of bFGF. Concentrations of heparin ranging from 0.4 μg/mL to 400 μg/mL resulted in minor but equivalent additional reduction in CPN dendritic complexity, axonal length and soma size. Thus, while heparin modestly potentiates the effects of bFGF on CPN morphology, heparin is not absolutely required for the observed effects of bFGF, and heparin’s effect on bFGF activity is not dose-dependent within the wide range investigated.

**The effects of bFGF directly counteract those of BDNF and NT-3, while those of BDNF and NT-3 are not additive**

The effects of bFGF counterbalanced those of BDNF and NT-3 (Fig. 5). The effects of BDNF + bFGF, NT-3 + bFGF or BDNF + NT-3 + bFGF were, for each parameter, intermediate between the effects of
bFGF alone and the effects of BDNF, NT-3, or BDNF + NT-3, respectively. Thus, even though all three peptides have effects that are appropriately considered 'trophic', these effects are specific, and appear to work at odds in determining more subtle aspects of cortical callosal neuron morphology, polarity and dendritic complexity. In contrast, the effects of BDNF and NT-3 were not additive (Fig. 5). Dendritic number and complexity in the presence of BDNF and NT-3 together were similar to the values in BDNF or NT-3 alone, while greater than the control values in SFM. Interestingly, BDNF and NT-3 together also resulted in a small decrease in axon length. This was surprising, as BDNF had no effect on axon length, and NT-3 had a small, non-significant effect.

**Observed effects of bFGF are not due to a cryptic mitotic population**

Because of bFGF’s well-established role as a key regulator of cortical precursor proliferation (Dotti et al., 1988; Ghosh & Greenberg, 1995; Vaccarino et al., 1999; Raballo et al., 2000) and its ability to inhibit cortical neurogenesis (Faux et al., 2001), we investigated whether our FACS-purified cultures might possibly contain a small, unrecognized population of dividing precursors or other cells that might confound our bFGF results (particularly if they were small, simple cells responsive to FGF as a mitogen), by labelling purified cells with the thymidine analogue BrdU and assessing proliferation in bFGF. Such
Fig. 5. The effects of basic fibroblast growth factor (bFGF) counteract those of brain-derived neurotrophic factor (BDNF) and NT-3, while those of BDNF and NT-3 are not additive. The most notable results are shown in a striped pattern. While the effects of BDNF and NT-3 are not additive, bFGF opposes the effects of either growth factor, alone or in combination. Asterisks denote values significantly different from serum-free medium (SFM). *P < 0.01; **P < 0.001.

Table 1. Summary of stage-specific effects of individual peptide growth factors on CPN morphology

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<th>BDNF</th>
<th>NT-3</th>
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<tr>
<td><strong>E19</strong></td>
<td>↓ axon length</td>
<td>↑ dendritic complexity</td>
<td>↓ dendritic number</td>
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<tr>
<td><strong>P2–3</strong></td>
<td>↑ dendritic number</td>
<td>↓ dendritic number</td>
<td>↓ dendritic complexity</td>
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<td></td>
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<td>↓ axon length</td>
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bFGF exhibits opposing effects to those of both BDNF and NT-3. BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; ↓, increased; ↓, decreased.

a cryptic population could potentially explain the relative small and simple morphology observed with bFGF. We have previously shown that purified CPN in CM do not divide, consistent with their identity as postmitotic neurons (Catapano et al., 2001). Cells in these cultures did not incorporate BrdU with or without bFGF (0% BrdU-positive vs. 20–25% BrdU-positive in control glial cultures), confirming that there are no mitotic cells present, that the bFGF effects observed are truly on pure postmitotic CPN, and that these effects are direct and specifically inhibitory for axonal and dendritic outgrowth.

**Effects on CPN morphology are independent of CPN survival**

To exclude the possibility that the observed effects of these growth factors on P2–3 CPN morphology were due to effects on cell viability or survival rather than specific effects on polarity and axonal/dendritic outgrowth, we employed mice with targeted deletion of the proapoptotic gene Bax (Knudson et al., 1995). Bax−/− neurons are protected from apoptosis, and Bax−/− sensory neurons survive in the absence of exogenous NGF, which is normally required for survival (Deckwerth et al., 1996; Patel et al., 2000). Similarly, we found that Bax−/− CPN survival in SFM was dramatically increased compared with wild-type CPN survival (approximately sevenfold). P2–3 Bax−/− CPN survival was not significantly different in BDNF, bFGF or CM, compared with SFM alone (91 ± 4% in BDNF; 92 ± 16% in bFGF; 92 ± 17% in CM; and 100 ± 4% in SFM, normalized to SFM survival), in contrast to the significantly increased survival of P2–3 wild-type CPN in CM or BDNF compared with SFM (Catapano et al., 2001). Consistent with these results, we found that purified P2–3 Bax−/− CPN in vitro exhibited significantly fewer apoptotic nuclei in SFM compared with wild-type P2–3 CPN (<1% in Bax−/− vs. 20–30% in wild-type) when nuclei were visualized with Hoechst, EthD-1 or propidium iodide.

We compared the effect of each growth factor on Bax−/− CPN with its effect on wild-type CPN to confirm that effects on morphology were not secondary to effects on CPN survival (Fig. 6). In CM, Bax−/− CPN displayed the same increase in axon length observed in wild-type CPN, and were on average slightly more complex than wild-type CPN. Similarly, BDNF, NT-3, bFGF and CNTF had qualitatively and quantitatively similar effects on Bax−/− CPN compared with their effects on wild-type CPN. In SFM, Bax−/− CPN were typically only slightly less complex than wild-type CPN, with slightly decreased dendritic number and complexity compared with wild-type CPN in SFM. Taken together, these results confirm that the effects on CPN morphology of these peptide growth factors are independent of any effects on CPN survival.

As a surrogate indicator of cell viability and differentiation, soma size of Bax−/− CPN was compared with that of wild-type CPN. Bax−/− CPN soma appearance and size at P2–3 were not significantly different from those of wild-type P2–3 CPN under any condition, suggesting equivalent health of the CPN populations from wild-type and Bax−/− mice (Fig. 6). Together with the results above, these experiments indicate that the growth factors used in this study have specific effects on morphologic differentiation of CPN that are independent from their effects on CPN survival (Catapano et al., 2001).

P2–3 and P6–7 CPN share similar peptide controls over polarity and process differentiation

To determine whether the observed effects on morphology, polarity, dendritic number and complexity, and axonal growth are stage-
specific, we performed similar analyses using CPN purified from P6–7 mice. At this age, CPN in SFM typically possess four–five dendrites, with an average dendrite length of 35 μm and an average axon length of 200 μm. Similar to its effects at P2–3, CM at P6–7 increased axon length, dendrite length and complexity, and soma size (Fig. 7). BDNF and NT-3 increased dendritic number and complexity, but had no effect on axonal or dendritic length, similar to the effects of BDNF and NT-3 on P2–3 CPN. bFGF, consistent with its effects at P2–3, significantly decreased dendritic number and complexity, axon length and soma size. CNTF had no significant effect on any of the morphological parameters at this age, consistent with the results with P2–3 CPN. These data are summarized in Table I and Fig. 9.

While subtly different, the effects of BDNF, NT-3, CNTF and bFGF on CPN morphology at P2–3 and P6–7 are, overall, quite similar, suggesting stability of their role in process differentiation, after initial establishment of polarity. Taken together, these results also support the specificity of the effects produced by each of the growth factors tested.

Growth factors exert distinct, stage-specific effects on CPN morphology at E19 vs. P2–3 and P6–7

To investigate whether these candidate growth factors exert the observed effects on morphologic differentiation earlier in development, and whether their effects are stage-specific, we analysed CPN purified from E19 mice. At this stage, CPN of deep layer V of cortex are just completing migration and have just extended their axons across the midline. At this stage, CPN are morphologically distinct from those at later stages (P2–3 and P6–7), with much simpler and less mature dendritic trees, and therefore might be expected to show different responses to the same set of candidate extracellular factors.

Consistent with this hypothesis, we found different effects of BDNF, NT-3 and bFGF on CPN at this stage, compared with P2–3 and P6–7.
At E19, CPN in SFM typically displayed three–five dendrites, with an average dendrite length of 25 µm and an average axon length of 60 µm. Compared with SFM, CM increased dendritic complexity and axon length, but had no effect on dendritic number or soma size (Fig. 8). In contrast to its effects at P2–3 and P6–7, BDNF exerted only a modest inhibitory effect on axon length, and had no effect on any other parameters. NT-3 modestly increased dendritic number and complexity, without effect on axon length.CNTF again had no significant effect on any of these parameters at this age.

bFGF decreased dendritic number and complexity, and axon length. In contrast to its effects with P2–3 and P6–7 CPN, bFGF had no effect on soma size of E19 CPN. As with bFGF’s effects at P2–3 and P6–7, these results indicate a specific inhibitory effect on dendrite number and complexity, and on axon length. These data are summarized in Table 1 and Fig. 9.

**Discussion**

The ability to study growth and neurotrophic factor controls over the directed differentiation of single neuronal lineages may be critical to attempts at cellular repair of CNS circuitry involving the selective degeneration and/or injury of specific neuronal populations. This may take the form of directing the differentiation of early multipotent neural precursors or neuronal precursors toward specific lineages, after inducing initial neuronal specification. This goal has been severely limited by the inability to isolate and purify most CNS neuronal...
subpopulations, especially populations of cortical projection neurons that are specifically vulnerable in a wide variety of neurodegenerative diseases and acquired ischaemic and traumatic injuries. This direction is also limited by a substantial lack of markers to distinguish different populations of neurons in the mammalian cerebral cortex. The investigations reported here are directed toward this goal of neuronal differentiation and replacement in the CNS.

We previously reported novel approaches to overcome these difficulties using FACS sorting to purify neurons of the specific cortical projection neuron lineage of CPN to 99.5% purity (Catapano et al., 2001). In that first report, we employed these approaches to investigate controls over the survival of CPN, and found stage-specific sets of survival-promoting neurotrophins and growth factors. The ability to isolate pure lineages at distinct stages of development and culture them in defined media further enables the study of the direct and potentially stage-specific effects of growth factors on the differentiation of specific neuronal subtypes and lineages, without the potentially intervening influence of either glia or other neuronal populations. This report focuses on this latter and independent aim of understanding controls over CPN differentiation, eventually toward control and manipulation of polarity, dendritic outgrowth and axonal elongation during neuronal integration into diseased cortex for cellular repair.

The studies presented here demonstrate the direct and stage-specific effects of peptide growth factors on distinct aspects of morphologic differentiation of the specific cortical neuron lineage of CPN. Exposure to BDNF and NT-3 results in increased dendrite number and complexity in postnatal CPN, whereas exposure to bFGF results in reduced dendritic number and complexity, and reduced axonal length.
The dendrite outgrowth-promoting effects of BDNF and NT-3 and the outgrowth-inhibiting effects of bFGF are additive and counteracting, suggesting that they are independent of each other and operate via distinct signalling pathways.

These in vitro data indicate that a combination of positive and negative regulators of neuronal polarity and process differentiation act collaboratively to direct CPN dendritic arborization, axon elongation and, ultimately, neuronal connectivity. Recognizing the counteracting effects of BDNF/NT-3 vs. bFGF could be critical to attempts to support and enhance dendritic differentiation, axonal connectivity and neuronal integration of precursors and immature neurons toward neuronal cellular repair of damaged CNS circuitry. For example, because both BDNF/NT-3 and bFGF have been put forward as promising factors for enhancing neurogenesis and/or incorporation of newborn neurons (Kuhn et al., 1997; Benraiss et al., 2001; Pencea et al., 2001; Barnabe-Heider & Miller, 2003), and because bFGF is a mitogen for endogenous precursors in the region of the subventricular zone, the hippocampal dentate gyrus subgranular zone and the parenchyma itself, it would be important to understand the effects of bFGF and BDNF/NT-3 given sequentially or simultaneously.

The studies presented here demonstrate that cortical projection neurons in culture following FACS purification retain stage-specific characteristics of morphology and display distinct morphologic effects in response to growth factors. While varying to a degree expected in an in vitro setting, they remain stereotypical in their polarity and progressive development of long projection neuron morphology to a remarkable extent. CPN in vivo display stereotypical pyramidal neuron morphology: large cell soma, prominent apical dendrite projecting toward the superficial layers of cortex, multiple basal dendrites and a long axon that extends 3–6 mm in early postnatal cortex and 7–10 mm in adult cortex. Purified CPN in vitro largely retain this characteristic morphologic, despite axotomy during purification. These pyramidal neuron morphologies become progressively larger and more complex as CPN mature from E19 to P6–7. Purified postnatal CPN project multiple, branched dendrites and axons that extend up to 1.5 mm, even in isolation from other neuronal populations. We isolated CPN from three distinct stages of development in mouse: (i) E19, when motor-sensory CPN axons first extend to the midline via the corpus callosum (Floeter & Jones, 1985); (ii) P2–3, when their axons begin to innervate contralateral cortex; and (iii) P6–7, when target innervation is complete and a subpopulation of cortical neurons undergo developmental cell death (Spreafico et al., 1995), presumably due in part to competition for limiting concentrations of peptide growth factors. These progressive stages of differentiation are representative of critical stages through which newly incorporated neurons will need to transit in order to incorporate into the diseased adult cortex for circuitry repair.

Newly incorporated neurons, whether derived from transplanted neuroblasts or precursors, or from induction of neurogenesis from endogenous precursors/stem cells, undergo similar progressive differentiation following appropriate migration. First, they must initiate axon extension toward distant targets. Next they must innervate specific targets. Third, they must consolidate innervation and mature their dendritic growth while surviving competition with existing neurons for typically limiting trophic support. Thus, though likely spread out over a longer window of time during adult neuronal replacement due to larger anatomic size and the distances involved, these three stages represent potentially critical periods during which exogenous growth factor guidance could greatly enhance attempts at neuronal repopulation and cellular repair.

Control or enhancement/restriction of fine dendritic growth and elaboration of branches, spines and synapses may also be critical to precise cellular intercalation into existent cortical circuitry by newly incorporated neurons toward cellular repair. Characteristic of developing neurons, these FACS-purified CPN extend short (5–10 μm), fine, unbranched processes from the cell soma. These minor processes, described by Dotti et al. (1988), are precursors of dendrites, which develop after axon specification. These dendritic precursors on FACS-purified CPN also possess numerous short, fine, lateral protrusions that are typically 4–10 μm in length with tapered ends, consistent with the morphology of filopodia (Harris, 1999). These filopodia are thought to be precursors of branches (Dailey & Smith, 1996) and spines (Ziv & Smith, 1996), and are capable of actively forming synaptic contacts with incoming axonal growth cones. Together, these data suggest that these cultures of FACS-purified CPN allow close analysis of even very subtle stage-stereotypical dendritic development that closely mimic developmental events during initial cortical development in vivo, and that may be important for circuit reconstruction in the post-developmental CNS. Because potential mechanisms and controls over neuronal replacement and integration in the adult might be expected to be at least partially common with those during initial development and circuit organization, understanding and manipulating such mechanisms and controls could be critical to appropriately enhancing and/or restricting dendritic growth and branching while integrating to repair circuitry.

It may be quite important to separate controls over survival and specific aspects of differentiation of newly incorporated neurons for the goal of cellular repair. We have shown previously (Catapano et al., 2001) that peptide growth factors, including BDNF and NT-3, but not bFGF or CNTF, exert direct and stage-specific control over CPN survival (summarized in Table 2). For this reason, here we have examined the effects of growth factors on CPN with a targeted deletion in the pro-apoptotic gene Bax, which is required for neuronal death due to trophic factor deprivation (Deckwerth et al., 1996). Bax−/− CPN display substantially decreased dependence on exogenous trophic support for survival.

Because it may be advantageous to deliver growth factors in a temporal sequence to optimize both survival and directed differentiation of newly incorporated neurons, knowledge about stage-specific changes in both survival and differentiation controls may be critical to optimize cortical circuitry repair. In previous experiments, we demonstrated that BDNF and NT-3 promote CPN survival at P2–3 and P6–7, but not E19. CPN express TrkB and TrkC, the primary receptors for BDNF and NT-3, respectively, at all three ages (Catapano et al., 2001).
In the current experiments, BDNF and NT-3 specifically increase primary dendrite number at P2–3 and P6–7. The specificity of these effects on one aspect of morphologic development argues against an indirect effect via neuronal health. The effects of BDNF and NT-3 on Bax−/− CPN morphology are quantitatively similar to their respective effects on wild-type CPN, further confirming that their effects are not secondary to an improvement in the survival of CPN with complex morphologies. Thus, these neurotrophins exert both stage-specific survival and dendritic growth effects.

It will also be very important to understand interactions between these neurotrophins and other peptide growth factors such as bFGF, implicated in aspects of proliferation of neural precursors/stem cells, neurogenic differentiation and neuronal survival. For example, bFGF has been found to expand populations of multipotent neural precursors, both in vitro and in vivo, and has been tested in rodent models for its ability to increase neuronal recruitment (Gensburger et al., 1987; Kuhn et al., 1997; Gage, 2000; Martens et al., 2002). Though bFGF may well have positive effects on each of precursor proliferation, neurogenic differentiation and neuronal survival, its interactions with other potentially applicable factors are not at all understood. Our current experiments demonstrate that bFGF exerts a specific effect to reduce soma size, dendrite number/complexity and axon length in wild-type CPN, at all three ages examined. The morphologic effects of bFGF directly counteract those of BDNF and NT-3, and the combination diminishes dendritic growth and complexity in a manner that would not be optimal after initial neuronal recruitment takes place and newly incorporated neurons are integrating to repopulate complex circuitry.

Several lines of evidence support the idea that bFGF exerts direct effects on neuronal morphology and process complexity, not secondary to a negative influence on neuronal health or adhesion. First, bFGF has no effect on average dendrite length, arguing against non-specific, indirect effects. Second, in all experiments, CPN were visualized with the vital dye calcine, eliminating the possibility that non-viable cells were inadvertently included in our analysis, skewing results. Finally, our previous experiments showed that bFGF does not decrease CPN survival at E19 and P2–3. Together, these data support the conclusion that bFGF exerts direct and specific effects on dendrite number/complexity and axon length, independent of neuronal survival and health. The implication is that these seemingly inhibitory effects on development of polarity could negatively impact integration of newly incorporated neurons if bFGF were present during the period of active dendritic and axonal differentiation and connectivity.

The experiments presented here are the first to examine the effects of BDNF, NT-3 and bFGF on a specific cortical neuron lineage, although several studies from other labs and our own have reported their effects on heterogeneous cortical pyramidal neuron morphology (McAllister et al., 1995; Grill et al., 1997; Horch et al., 1999; Lom & Cohen-Cory, 1999; Gates et al., 2000; Xu et al., 2000; Yacobian & Lo, 2000). However, these experiments could not determine whether these effects were cell-autonomous or lineage-specific in distinct populations of cortical projection neurons vulnerable to neurodegenerative diseases or injury. Cellular repopulation and circuit repair would require precise formation of distinct neuronal subtypes. Thus, the current results, and the broader set of experiments they enable regarding other lineages of projection neurons, have the potential to contribute to the directed late-stage and lineage-specific differentiation of newly incorporated neurons following transplantation or in situ manipulation of neural precursors/stem cells in the adult brain.

Effects on axonal and dendritic differentiation of desired lineages of projection neurons by mitogenic growth factors often used in experiments manipulating endogenous and in vitro expanded neural precursors/stem cells may be of particular importance with regard to cellular repair. bFGF (along with epidermal growth factor) is a major mitogen for the populations of neural precursors known to exist in the adult mammalian subventricular zone, hippocampal dentate gyrus subgranular zone and at much lower frequency perivascularly within the parenchyma (Reynolds & Weiss, 1992; Richards et al., 1992; Palmer et al., 1995, 1999; Kuhn et al., 1997). Thus, potential undesired effects on differentiation of specific lineages by bFGF infused or applied ex vivo could be critical. The effect of bFGF on neurite outgrowth has been described in a number of CNS neurons (Hatten et al., 1988; Aoyagi et al., 1994; Lowenstein & Arsenault, 1996; Shitaka et al., 1996; Pataky et al., 2000), including neurons in neocortex (Walicke, 1988; Szebenyi et al., 2001). Szebenyi et al. (2001) described the effect of bFGF, applied to cortical slice cultures by attachment to latex microspheres, in promoting axon branching. Interestingly, and consistent with our data, the authors report that at the point where FGF-coated beads collected near an axon, axon elongation was inhibited and branching resulted. Other reports have similarly described axon branching at a site of growth cone pause (Halloran & Kalil, 1994; Szebenyi et al., 1998). Taken together, these findings suggest that environmental factors that halt axon elongation, as bFGF does in our experiments, may play an important role in the regulation of axon branching and target innervation, and could negatively affect connectivity of newly incorporated neurons during attempts at cellular repair of cortical projection neuron circuitry if applied at the wrong stage of the process. The promotion of neurite outgrowth by growth factors including BDNF and NT-3 has been extensively studied; however, much less is known about the potential mechanisms by which growth factors such as bFGF limit neurite outgrowth. In early neural precursor development, bFGF both stimulates proliferation and inhibits differentiation, the latter via the up regulation of Notch expression (Faux et al., 2001). Notch has also been shown to restrict neurite outgrowth in cortical neurons (Sebastian et al., 1999; Redmond et al., 2000). Together, these data raise the possibility that bFGF inhibition of neurite outgrowth is mediated by Notch, consistent with one of the central mechanisms by which bFGF regulates early neural precursor development.

It is still unknown whether these growth factors will similarly or distinctly affect other lineages of cortical neurons and, in particular, other lineages of projection neurons for repopulation of long-distance circuitry. This approach of purifying projection neuron lineages on the basis of axonal projections by FACS will allow future experiments to address such questions of specificity by comparing effects on distinct types of cortical projection neurons. While it is very likely that similar effects may be identified for multiple types of projection neurons, it is also likely that each unique population will be found to have subtly
Stage-specific controls over projection neuron differentiation


stem cells from diverse regions of the adult CNS. J. Neurosci., 19, 8487–8497.