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The Actions of Bone Morphogenetic Protein 2 on Mammalian Central Nervous System Stem Cells and Stem Cell Progeny

by

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Abstract

Bone morphogenetic protein 2 (BMP-2) is a member of the transforming growth factor ß superfamily of signaling molecules and has recently been described as an important molecule in many developmental processes. Our laboratory isolated a central nervous system (CNS) stem cell from the embryonic day 14 (E14) mouse striatum that will proliferate in the presence of epidermal growth factor (EGF) to form a cluster, or sphere, of undifferentiated cells. The stem cell progeny can differentiate into the three principal phenotypes of the CNS - neurons, astrocytes and oligodendrocytes. My results demonstrate that BMP-2 has at least two effects on CNS stem cells and their related progeny: 1) BMP-2 inhibits the proliferation of CNS stem cells cultured in the presence of EGF and 2) BMP-2 enhances the differentiation, but not the survival or the proliferation, of neurons isolated from cultures of dissociated spheres. Based on these results, coupled with the observation that BMP-2 and its receptor are present in the E14 striatum, I conclude that BMP-2 likely plays an active role in E14 striatal neurogenesis by regulating precursor proliferation and by enhancing neuronal differentiation from undifferentiated precursor cells.

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To

Marg, Lib, Trevor and Poco

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Abbreviations Used

ActR-I	type I activin receptor
ALK-3	activin-like kinase 3
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BMP-2	bone morphogenetic protein 2
BMP-2R	bone morphogenetic protein 2 receptor
BMP-4	bone morphogenetic protein 4
BMPR-IA	bone morphogenetic protein receptor type IA
BMPR-IB	bone morphogenetic protein receptor type IB
BRK-1	bone morphogenetic protein receptor kinase 1
BrdU	bromodeoxyuridine
CNS	central nervous system
ddH_2O	double distilled water
DIV	days *in vitro*
DMEM	Dulbecco's modified Eagle medium
dNTP	deoxynucleotide triphosphate
E14	embryonic day 14
EGF	epidermal growth factor
FGF	fibroblast growth factor
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GGF	glial-derived growth factor
HEPES	N-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
MIP-1α	macrophage inflammatory protein 1α
MPC	mitotically active precursor cell
NCAM	neural cell adhesion molecule

NGS	normal goat serum
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNS	peripheral nervous system
RDP	rapid differentiation protocol
RNA	ribonucleic acid
RPM	rotations per minute
RT	reverse transcriptase
TGF-ß	transforming growth factor ß
TGF-ß1	transforming growth factor ß1
TSC	totipotent stem cell

1 INTRODUCTION

1.1 Stem Cells

In most mammalian tissues, there is a continual replacement of dead or dying cells throughout the lifetime of the animal. This replacement can occur by one of two mechanisms: 1) the division of a cell which produces two identical daughters or 2) the production of a variety of new cells from a single precursor commonly termed a stem cell (Potten, 1986; Alberts *et al.*, 1994). Stem cells are functionally defined as "undifferentiated cells capable of, a) proliferation, b) self-maintenance, c) the production of a large number of differentiated, functional progeny, d) regenerating the tissue after injury, and e) a flexibility in the use of these options" (Potten and Loeffler, 1990). According to this definition, stem cells are involved in homeostatic processes and therefore exist only in the adult animal (Hall and Watt, 1989; Price, 1995). Yet, cells that possess some stem cell-like characteristics are present in the embryo as well (Hall and Watt, 1989).

The primary function of a totipotent stem cell (TSC, sometimes called a pluripotent stem cell) is to produce every cell type found within a system/tissue. By mechanisms that are not entirely understood, the TSC divides asymmetrically to produce two cells - another TSC and a mitotically active precursor cell (MPC; Hall and Watt, 1989; Potten and Loeffler, 1990; Morshead *et al.*, 1994; Dzierzak and Medvinsky, 1995). The MPC continues to divide, producing all of the cell types of the tissue while the TSC remains mitotically quiescent. The survival and proliferation of both TSCs and MPCs is under the control of various intrinsic and extrinsic factors.

To date, stem cells have been best characterized in the intestinal crypts, the bone marrow, various epithelia (Potten, 1986; Hall and Watt, 1989; Potten and Loeffler, 1990; Alberts *et al.*, 1994) and the mammalian central nervous system (CNS; Weiss *et al.*, 1996).

1.2 Stem Cells of the Central Nervous System

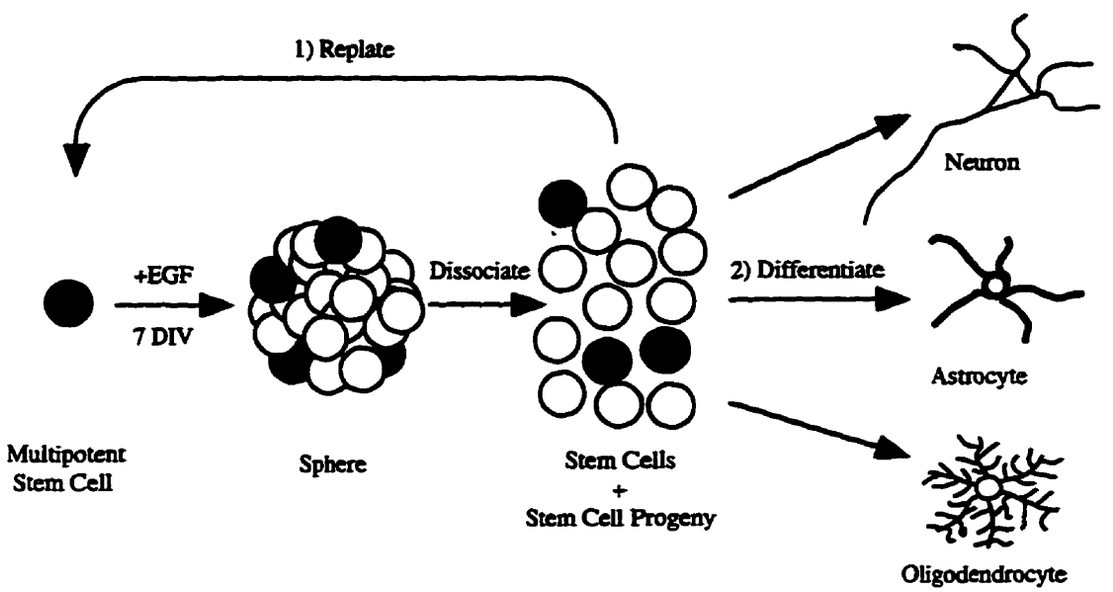
Our laboratory has identified a cell in the embryonic (Reynolds *et al.*, 1992) and adult (Reynolds and Weiss, 1992) mouse striatum that proliferates in response to epidermal growth factor (EGF) *in vitro* to form a cluster, or sphere, of undifferentiated cells. When allowed to differentiate on poly-ornithine-coated coverslips, cells within the spheres are capable of differentiating into the three major cell types found in the adult mammalian CNS - neurons, astrocytes and oligodendrocytes (Figure 1). When spheres are dissociated into single cells and replated into culture medium supplemented with EGF, secondary spheres form, indicating that these cells are capable of both proliferation and self-maintenance (Figure 1). In the adult, these cells are capable of proliferating *in vivo* (Morshead *et al.*, 1994; Craig *et al.*, 1996) and may be the source of new neurons in the olfactory bulb (Lois and Alvarez-Buylla, 1994). Thus, many of the criteria outlined in section 1.1 have been satisfied, thereby supporting the presence of a stem cell in the mammalian CNS (for review see Weiss *et al.*, 1996).

There are at least two populations of cells found within EGF-generated spheres. Stem cells that proliferate to form secondary spheres (20%; Reynolds and Weiss, 1996), and stem cell progeny that can both proliferate and differentiate (Vescovi *et al.*, 1993; Reynolds and Weiss, 1996) into the three principal cell types of the CNS (80%) yet cannot form spheres. It is still unclear how TSCs and MPCs may operate in CNS stem cell populations, though experimental evidence suggests that both may be present (Morshead and van der Kooy, 1992; Morshead *et al.*, 1994). To date, only EGF (Reynolds and Weiss, 1992) and basic fibroblast growth factor (bFGF; Gritti *et al.*, 1996) have been identified as proliferative agents for CNS stem cells. With regards to differentiation, brain-derived neurotrophic factor (BDNF) is the only molecule identified to date with the ability to influence the phenotypic fate of cells within spheres (Ahmed *et al.*, 1995).

Figure 1:

Schematic representation of EGF-responsive stem cell proliferation and the differentiation of stem cell progeny.

A multipotent stem cell is isolated from the embryonic day 14 mouse striatum and plated in EGF-containing culture medium. After 7 days *in vitro*, a clonally-derived sphere is formed. This sphere can be dissociated into single cells and either 1) replated back into EGF to produce more spheres or 2) plated on poly-ornithine coated glass coverslips to induce the differentiation of stem cell progeny.


1) Replate
Neuron
+EGF
7 DIV
Dissociate
2) Differentiate
Astrocyte
Multipotent
Stem Cell
Sphere
Stem Cells
+
Stem Cell Progeny
Oligodendrocyte

1.3 Hematopoiesis and Neuropoiesis

Compared to stem cells from other systems, very little is known about stem cells isolated from the CNS. The identification of factors that regulate the growth and differentiation of CNS stem cells and stem cell progeny is still in its infancy. For example, only EGF and bFGF have been identified as mitogens for CNS stem cells (Reynolds *et al.*, 1992; Gritti *et al.*, 1996). Therefore, there are two principal issues that concern our laboratory: 1) how is the proliferation of CNS stem cells regulated and 2) what are the epigenetic factors that control the differentiation of stem cell progeny? It is instructive to consider hematopoiesis and peripheral nervous system (PNS) neuropoiesis since similar processes may govern the proliferation and fate of CNS stem cells and stem cell progeny.

Hematopoiesis occurs in the bone marrow and spleen of adult vertebrates and is responsible for the production of large numbers of all differentiated blood cell types (Hall and Watt, 1989; Alberts *et al.*, 1994). These include erythrocytes (red blood cells), leukocytes (granulocytes, monocytes, lymphocytes and natural killer cells) and platelets (Alberts *et al.*, 1994). Hematopoiesis is regulated by a multitude of soluble factors present in the blood plasma, collectively referred to as cytokines (Ogawa, 1993). In hematopoiesis, a TSC will divide asymmetrically to produce one of itself and one MPC (Mayani *et al.*, 1993). Once an MPC is produced, all aspects of its survival, proliferation and maturation are controlled by cytokines. A very important point to emphasize is that the progenitor cell produced is committed to a specific lineage (Ogawa, 1993). This is the first and only determinant step in the differentiation process. As a consequence, cytokines act permissively rather than instructively to promote the development of a precursor into a terminally-differentiated cell (Metcalf, 1989; Fairbairn *et al.*, 1993; Ogawa, 1993). Cytokines do not determine what a cell is to become; rather, cytokines determine which precursors survive (Fairbairn *et al.*, 1993).

Neuropoiesis, the process suggested to govern the genesis of different neural crest cell types during development, was first proposed by Anderson (1989). Neuropoiesis is the process of gradual, stepwise lineage restriction starting with an undifferentiated stem cell and ending with the fully-differentiated cell types found in the PNS. Interestingly, a PNS stem cell was not identified until well after the neuropoietic model was put forth (Stemple and Anderson, 1992).

Just how extrinsic factors influence the fate of neural crest precursors has only recently been addressed. Earlier this year, Shah *et al.* (1996) demonstrated that neural crest stem cells responded quite differently to members of the transforming growth factor ß (TGF-ß) superfamily to produce three different cell types. It was found that in the presence of glial-derived growth factor (GGF), transforming growth factor ß1 (TGF-ß1) and bone morphogenetic protein 2 (BMP-2), PNS stem cells would generate a homogenous population of astrocytes, smooth muscle cells or neurons, respectively (Shah *et al.*, 1996). Since each factor favored the production of one cell type at the expense of the others, without regulating survival, these factors were deemed to act instructively rather that permissively (Shah *et al.*, 1994, 1996).

The regulation of CNS stem cells may be governed by a process similar to hematopoiesis and neuropoiesis. A representation of this hypothetical CNS stem cell hierarchy is illustrated in Figure 2.

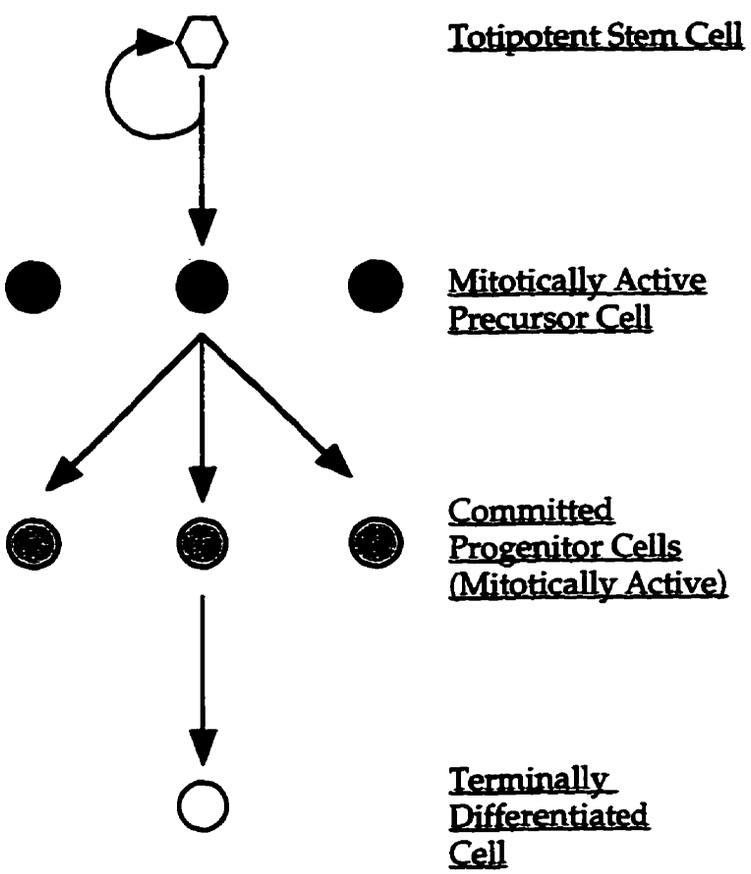
1.4 Mechanisms/Processes that May Mediate Extrinsic Factor Enhancement of Neural Numbers from Stem Cell Progeny

As described in section 1.3, extrinsic or epigenetic factors may regulate the production of different cell types from multipotent precursors. To accomplish this, extrinsic factors may employ a number of mechanisms/processes. However, just how and where in the lineage process these operate is complex. Therefore, in an attempt to simplify this issue, I

Figure 2:

Hypothetical representation of CNS stem cell lineage based on hematopoiesis and neuropoiesis.

A totipotent stem cell is capable of producing all of the different cell types found within the adult CNS. However, the TSC does not directly produce these different cell types. An MPC is generated through an asymmetric division of the TSC. The MPC will produce a committed progenitor cell, and the committed progenitor cell will give rise to a fully differentiated cell. The differentiated cell can be either a neuron, an astrocyte or an oligodendrocyte.


Totipotent Stem Cell
Mitotically Active Precursor Cell
Committed Progenitor Cells (Mitotically Active)
Terminally Differentiated Cell

propose that there are three principal mechanisms/processes whereby epigenetic factors can increase cellular numbers: proliferation, survival and differentiation. For clarity, these mechanisms are defined as follows:

1.4.1 Proliferation

Proliferation is defined as "...producing new cells. With each mitosis there is a net gain of one cell" (Lajtha, 1979) or, according to the Oxford dictionary (Hawkins and Allen, 1991), "to reproduce; increase rapidly in numbers; grow by multiplication". These definitions have been expanded to "a process involving a sequential pattern of (cyclic, repeating) changes in gene expression leading ultimately to the physical division of cells" (Potten and Loeffler, 1990).

In the CNS, increases in cellular numbers may be epigenetically regulated through proliferation. Two examples of this are EGF (Anchan *et al.*, 1991; Lillien and Cepko, 1992; Reynolds *et al.*, 1992; Reynolds and Weiss, 1992, 1996) and bFGF (Gensburger *et al.*, 1987; Murphy *et al.*, 1990; Kilpatrick and Bartlett, 1993; Ray *et al.*, 1993; Vescovi *et al.*, 1993; Ray and Gage, 1994; Palmer *et al.*, 1995) which increase the numbers of neurons and glia from precursor cells of the CNS. Using this information, I have defined a proliferation factor as follows:

Proliferation Factor	A factor that promotes the (re-)entry of a cell into the cell cycle.

1.4.2 Survival

The best description of survival is the prevention of cellular death. There is a group of molecules collectively referred to as neurotrophins that have been well characterized for their ability to prevent neuronal cell death (Levi-Montalcini, 1987; Davies, 1994). Trophic literally means "of or concerned with nutrition". In other words, neurotrophins are food and

without food cells would die. Extrinsic factors such as leukemia inhibitory factor, ciliary neurotrophic factor , BDNF, neurotrophins-3, 4/5 and 6 as well as selected members of the fibroblast growth factor (FGF) family have neurotrophic effects on a variety of neurons isolated from both the PNS and the CNS (for reviews see Korsching, 1993 and Davies, 1994). A recently discovered member of the TGF-ß superfamily of molecules, glial cell line-derived neurotrophic factor, is capable of supporting the survival of neurons from the CNS (Lin *et al.*, 1993).

Though the actions of neurotrophins are not restricted to the survival of cells in the nervous system, experimental evidence suggests that they must be continuously present to exert their effect (Alderson *et al.*, 1990; Hyman *et al.*, 1991; Knusel *et al.*, 1991). Therefore, my definition of a survival factor is as follows:

Survival Factor — A factor whose continual presence is required by a cell for it to avoid death by either necrosis or apoptosis.

1.4.3 Differentiation

Differentiation has been defined as a "process by which a cell undergoes a change to an overtly specialized cell type" (Alberts *et al.*, 1994). More specifically, differentiation involves "a qualitative change in the cellular phenotype that is the consequence of the onset of synthesis of new gene products" (Potten and Loeffler, 1990). This idea was first postulated by Monod and Jacob who stated that "cell differentiation is achieved by the activation of specific sets of genes, with each specific cell type expressing a different set of genes" (paraphrased by Jessel in Kandel *et al.*, 1995). Thus, the following definition is proposed:

Differentiation Factor — A factor that promotes/allows a precursor cell to develop into a more mature phenotype.

Schematically, these three mechanisms/processes are illustrated in Figure 3 which also outlines the experimental paradigms used in sections 4.1.2 to 4.1.4.

1.5 Bone Morphogenetic Protein 2

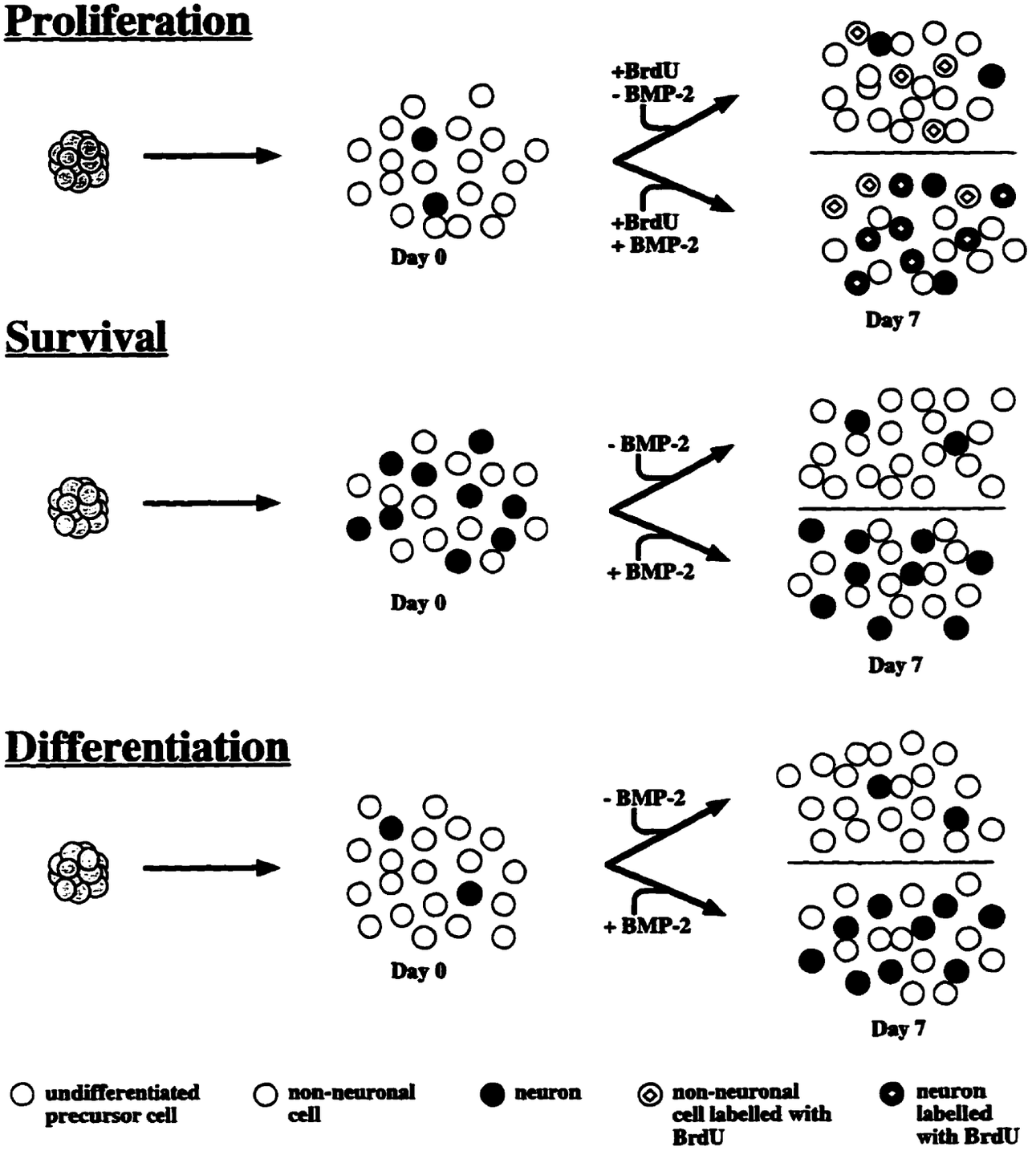
BMP-2 is a member of the decapentaplegic / vg-1 subfamily of the TGF-ß superfamily of molecules (Kingsley, 1994; Massague *et al.*, 1994). BMP-2 and bone morphogenetic protein 4 (BMP-4), which share 92% amino acid homology, are considered to be the mammalian homologues of the *Drosophila* gene *decapentaplegic* (Padgett *et al.*, 1987; Gelbart, 1989). BMP-2 was first isolated from bovine bone (Sampath and Reddi, 1981) and later identified as an inducer of ectopic bone formation in embryonic rats (Wozney *et al.*, 1988). Subsequent studies have identified specific roles for BMP-2 in the development of the chick limb bud (Niswander and Martin, 1993; Francis *et al.*, 1994; Tickle and Eichele, 1995; Duprez *et al.*, 1996; Zou and Niswander, 1996), as well as in other osteogenic processes (Wozney, 1992; Hughes *et al.*, 1995).

The actions of bone morphogenetic proteins (BMPs) are not, however, restricted to osteogenic processes, as they are important during many other developmental processes. This is suggested by the ubiquitous distribution of the BMP-2 receptor, BMP receptor type IB (BMPRIB; Mishina *et al.*, 1995) and by the embryonic lethality of BMP-2 (Zhang and Bradley, 1996) and BMP-4 knockouts (Winnier *et al.*, 1995). BMPs are directly involved in the ventralization of *Xenopus* embryos (Harland, 1994; Clement *et al.*, 1995; Hawley *et al.*, 1995; Northrop *et al.*, 1995; Jones *et al.*, 1996) in concert with *chordin* (Sasai *et al.*, 1995; Piccolo *et al.*, 1996) and/or *noggin* (Re'em-Kalma *et al.*, 1995; Zimmerman *et al.*, 1996). In the mouse, BMPs are required for proper mesodermal induction (Mishina *et al.*, 1995; Winnier *et al.*, 1995) and in the chick, BMPs promote neural induction in sequence with *dorsalin*

Figure 3:

Mechanisms/processes that an extrinsic factor may employ to regulate the production of a specific neural cell type.

There are three mechanisms/processes that could be used by a growth factor to increase neural cell numbers. A few hypothetical experimental outcomes are also illustrated. See sections 4.1.2 to 4.1.4 for a description of the experimental procedures used to test such mechanisms/processes.


Proliferation
+BrdU
- BMP-2
+BrdU
+ BMP-2
Day 0
Day 7
Survival
- BMP-2
+ BMP-2
Day 0
Day 7
Differentiation
- BMP-2
+ BMP-2
Day 0
Day 7
undifferentiated precursor cell
non-neuronal cell
neuron
non-neuronal cell labelled with BrdU
neuron labelled with BrdU

(Liem *et al.*, 1995). BMPs are also involved in later embryologic events such as the development of the kidney (Dudley *et al.*, 1995; Luo *et al.*, 1995), heart (Zhang *et al.*, 1996; Winnier *et al.*, 1995), lung (Bellusci *et al.*, 1996) and PNS (Shah *et al.*, 1996). For a recent, comprehensive review see Hogan (1996).

1.6 Receptors for BMP-2

Members of the TGF-ß superfamily can bind to three types of receptors: type I, II and III (Kingsley, 1994; Massague *et al.*, 1994). The type III receptor is a membrane bound proteoglycan (Wang *et al.*, 1991) implicated in presenting TGF-ß ligands to the type I and II receptors (Lopez-Casillas *et al.*, 1993). This is analogous to heparan sulfate proteoglycan function in FGF signaling (Spivak-Kroizman *et al.*, 1994). Unlike the type III receptor, the type I/type II receptors are both serine/threonine kinases that must form a heterodimer to transduce a signal across the membrane. To achieve this, the type II receptor binds to its respective ligand thereby allowing the type II receptor to bind to and activate the type I receptor. This heterodimer becomes an active complex that transduces a signal across the membrane (Wrana *et al.*, 1994). There are three identified type I receptors that can be activated in the presence of BMP-2 (Massague, 1996): BMP receptor kinase 1 (BRK-1 or bone morphogenetic protein receptor type IA (BMPR-IA); Koenig *et al.*, 1994), bone morphogenetic protein 2 receptor (BMP-2R or BMPR-IB Mishina *et al.*, 1995) and the type I activin receptor (ActR-I or activin like kinase 3 (ALK-3); ten Dijke *et al.*, 1994). Although a mouse type II receptor has yet to be cloned, BMP-2 can bind to the gene product of *daf-4* from *C. elegans* (Estevez *et al.*, 1993) and BMP receptor II from humans (Liu *et al.*, 1995; Rosenzweig *et al.*, 1995). Evidence suggests that there are many different type I/type II receptor combinations possible (Matzuk *et al.*, 1995), which accounts for the diverse number of actions seen for one ligand (Liu *et al.*, 1995; Matzuk *et al.*, 1995; Massague, 1996).

1.7 Actions of BMPs during Neural Development

Although the distribution of BMP-2 has been described during the early development of both the mouse (Lyons *et al.*, 1989, 1990, 1995) and frog (Clement *et al.*, 1995), its precise location in the developing CNS has not been well described. Transcripts for the BMP receptor BRK-1 have been localized to the neural layer of the retina and the ependymal layers of the different ventricles (DeWulf *et al.*, 1995). Furthermore, no study has identified a role for BMP-2 during CNS development *in vivo*, though BMPs have been shown to enhance the induction of various neurotransmitter synthesizing enzymes in cultured rat sympathetic neurons (Fann and Patterson, 1994). BMPs can also upregulate the production of the cell adhesion molecules L1 and neural cell adhesion molecule (NCAM) from PC12 cells *in vitro* (Paralkar *et al.*, 1992; Perides *et al.*, 1994). Recently, it has been demonstrated that BMPs can upregulate both neuronal production from PNS stem cell progeny (Shah *et al.*, 1996) and increase production of the astroglial lineage from EGF-generated spheres (Mehler *et al.*, 1995; Gross *et al.*, 1996). Thus, despite its presence in the embryonic CNS neural primordia, relatively little is known about the role of BMP-2 in neural cell histogenesis.

1.8 Statement of Hypothesis

BMP-2 regulates the proliferation of embryonic CNS stem cells and the differentiation of stem cell progeny.

1.9 Experimental Objectives

i) Does BMP-2 regulate the proliferation of EGF-responsive neural stem cells isolated from the E14 mouse striatum?

The identification of epigenetic factors that regulate the proliferation of CNS stem cells is of great importance. To determine if BMP-2 could influence stem cell proliferation, the stem cell proliferation assay was used. Primary cultures were plated into 96-well plates in the absence or presence of increasing concentrations of BMP-2. Since each sphere produced after 10 days *in vitro* (DIV) is representative of the number of stem cells present at plating, the appearance or absence of spheres after 10 DIV was monitored.

ii) Does BMP-2 influence the fate of CNS stem cell progeny?

CNS stem cell progeny are undifferentiated while in the sphere, yet differentiate into the three principal CNS cell types when dissociated from a sphere and plated. Because of this, they present an attractive system for the identification of epigenetic factors that may influence cell fate in the CNS. To test the action of BMP-2 on CNS stem cell progeny, the rapid differentiation protocol (RDP) was used. Dissociated pass-1 spheres were plated onto poly-ornithine-coated coverslips in the absence or the presence of BMP-2. After 7 DIV, indirect immunocytochemistry was used to monitor the appearance of the various cell types found in the CNS.

iii) Does BMP-2 increase neural cell numbers through a mechanism of proliferation, survival and/or differentiation?

If BMP-2 regulates the appearance of a specific neural phenotype, then the outcome of four experiments should determine whether it acts as a proliferative, a survival or a differentiation factor for CNS stem cell progeny. These three mechanisms/processes, and how they regulate cell numbers, are described in section 1.4 and illustrated in Figure 3. Cells plated under RDP conditions will be subject to different manipulations to delineate the mechanism(s) used by BMP-2 to regulate neural numbers. Cells will be detected after 7 DIV by immunocytochemical methods.

2 METHODS AND MATERIALS

2.1 Cell Culture

Striata from embryonic day 14 (E14) CD1 albino mouse embryos (Charles River) were removed and dissociated with a fire-narrowed Pasteur pipette in culture medium. The culture medium used was DMEM/F12 (1:1; Gibco) supplemented with glucose (0.6%), glutamine (2 mM), sodium bicarbonate (3 mM), HEPES buffer and a hormone mix supplement comprised of insulin (25 µg/mL), transferrin (100 ng/mL), progesterone (20 nM), putrescine (60 nM) and selenium chloride (30 nM) (all from Sigma except glutamine; Gibco). Cells were plated at a density of 200 000 cells/mL in Corning T75 culture flasks in 40 mL of the described culture medium with 20 ng/mL EGF (generously supplied by Chiron). The anchorage independent clusters of cells that form after 7 DIV are called primary spheres.

To passage primary spheres, 7 day old cultures were centrifuged at 400 rotations per minute (RPM) for 10 minutes, resuspended in 5 mL of culture medium, dissociated by trituration with a fire-narrowed Pasteur pipette and replated at a density of 50 000 cells/mL in EGF-containing culture medium. The spheres that form after 7 DIV are called pass-1 spheres.

2.2 BMP-2

My first source of human recombinant BMP-2 was from Chiron (Emeryville, CA, USA). Once this supply was exhausted, I switched to Ciba Geigy (Basel, Switzerland) as my new source since I was unable to obtain any more reliable samples from Chiron. I discovered that the two sources produced indistinguishable cellular effects, however 3 times more Ciba Geigy BMP-2 than Chiron BMP-2 was required to obtain the same results. All of the experiments described in this thesis were performed using Ciba Geigy BMP-2 except for the experiments outlined in sections 3.1.3 and 3.1.5.

2.3 Stem Cell Proliferation Assay

To measure the proliferation of stem cells, cells isolated from the murine E14 striatum were plated at a density of 25 000 cells/mL (5000 cells/well) in 96-well plates (Nunclon) with culture medium containing EGF (20 ng/mL). Human recombinant BMP-2 (generously supplied by Ciba-Geigy and Chiron) was added to each well using a 10 µL aliquot of the desired concentration. The number of spheres per well was quantified after 10 DIV (8 wells per condition, n=at least 3 independent cultures).

2.4 Rapid Differentiation Protocol

Pass-1 EGF-generated spheres were rinsed free of EGF containing culture medium, dissociated by trituration with a fire-narrowed Pasteur pipette and plated at a density of 200 000 cells/mL onto poly-ornithine (150 µg/mL; Sigma) coated glass coverslips in 24-well plates (Nunclon) with culture medium. No fetal calf serum or EGF was added. Total cell numbers were quantified by counting nuclei counterstained with bis-benzamide. All other cell types were identified using immunocytochemical methods.

2.5 Antibodies

Mouse monoclonal antibodies and rabbit polyclonal antisera were used for indirect immunocytochemistry. Primary antibodies included monoclonal ß-tubulin (type III, IgG, 1:1000; Sigma), bromodeoxyuridine (BrdU, IgG, 1:5; Amersham) and O4 (IgM, 1:20; Boehringer Manheim) and polyclonal antisera to γ-aminobutyric acid (GABA, 1:8000; Sigma) and glial fibrillary acidic protein (GFAP, 1:400; Biomedical Technologies Inc.). Rhodamine isothiocyanate-conjugated affinipure goat antibody to mouse IgG (1:200), fluorescein isothiocyanate-conjugated affinipure goat antibody to rabbit IgG (1:100) or fluorescein isothiocyanate-conjugated affinipure goat antibody to mouse IgM

(1:100) were used as secondary antibodies (all from Jackson Immunochemicals).

2.6 Indirect Immunocytochemistry

The standard protocol for single label indirect immunocytochemistry is as follows. RDP coverslips were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) (+0.1% gluteraldehyde for anti-GABA immunocytochemistry) for 20 minutes at room temperature and then washed 3 times (10 minutes each) with PBS. Primary antibodies were added to each coverslip in a solution of 10% normal goat serum (NGS; Jackson Immunochemicals)/0.3% Triton-X100 (Gibco) in PBS and incubated for 2 hours at 37°C. Coverslips were washed 3 times with PBS then the secondary antibody was added and incubated for 30 minutes at room temperature. Cells were washed 3 times with PBS, incubated with the nuclear stain, bis-benzamide (1:100; Sigma) for 20 minutes at room temperature, washed 2 more times with PBS then mounted on glass slides using Fluorsave (Calbiochem) as the mounting medium. Cells were observed and photographed using a Zeiss photomicroscope III.

For reactions involving the O4 antibody, no permeablization with Triton X-100 was used. In the case of ß-tubulin and BrdU double labeling, the protocol outlined above is repeated, first using the BrdU antibody, then with the ß-tubulin antibody. Anti ß-tubulin antibody (the second antibody) was added in 1% NGS.

2.7 Reverse Transcription-Polymerase Chain Reaction

Total ribonucleic acid (RNA) from both the E14 mouse striatum and pass-1 spheres was extracted using TRIzol® reagent (Gibco) according to the manufacturer's specifications. Briefly, a pellet of tissue was suspended thoroughly in the appropriate amount of TRIzol reagent (1 mL/1.0X10^7 cells)

and incubated at room temperature for 5 minutes. Chloroform (0.2 volumes) was added, the suspension was mixed thoroughly and then it was centrifuged at 10 000 RPM for 10 minutes. The aqueous phase was removed, an equal volume of isopropanol was added and the solution was left overnight at -20°C. The solutions were spun down at 10 000 RPM, the supernatant was removed and the pellet was dissolved in 100 µL 4M guanididium isothiocyanate. Two volumes of 95% ethanol were added and the solution was incubated overnight at -80°C. The solution was centrifuged at 10 000 RPM for 10 minutes, the supernatant was removed and the pellet was washed with 70% ethanol. The final pellet was resuspended in double distilled H_2O (ddH_2O). One microgram of total RNA from both samples were added to 0.5 µg oligo $(dT)_{12-18}$ (Gibco) and incubated for 10 minutes at 65°C. SuperScript reverse transcriptase (RT; 200 units, Gibco) and deoxynucleotide triphosphates (dNTPs; 10 mM in each) were added, the solution was incubated for 90 minutes at 42°C, then diluted 1.5X with ddH_2O. RT product (3%) was used in 40 cycles of the polymerase chain reaction (PCR) using the following conditions: 1 minute at 94°C (separation), 1 minute at 58°C (annealing) and 1 minute at 72°C (extension). All PCR solutions contained Taq polymerase (2.5 U), $MgCl_2$ (1.5 mM), dNTPs (10 mM in each), PCR buffer (Gibco) and the appropriate primers (0.25 pmol of each), all in ddH_2O. The BMP-2 product was amplified using 10% glycerol. The primers used were as follows: 1) for the BMP-2 ligand (GENBANK accession #L25602) the 5′ (sense) primer corresponded to bases 591-614 and the 3′ (antisense) primer to bases 1104-1127, 2) for BRK-1 (GENBANK accession #U04672) the 5′ primer corresponded to bases 841-860 and the 3′ primer to bases 1134-1153, and 3) for ß-actin (GENBANK accession #X03672) the 5′ primer corresponded to bases 182-202 and the 3′ primer to bases 403-424. PCR products were analyzed on a 1.5% agarose gel with 12.5 ng/mL ethidium bromide and photographed using a Polaroid camera.

2.8 Statistical Methods

Data was analyzed by first using an analysis of variance to determine significance. Tukey's honestly significantly different post-hoc test was used to determine where variance(s) occurred in the sample using a threshold of significance of $p<0.05$.

3 BMP-2 INHIBITS THE PROLIFERATION OF EMBRYONIC CNS STEM CELLS CULTURED IN THE PRESENCE OF EGF

3.1 RESULTS

3.1.1 Transcripts for BRK-1 are present in the E14 striatum and in EGF-generated spheres whereas BMP-2 transcripts are present only in the E14 striatum

Before analyzing the action(s) of BMP-2 on CNS stem cells, the expression of both BMP-2 and one of its type I receptors, BRK-1, was examined using RT-PCR. PCR primer pairs specific for BMP-2, BRK-1 and ß-actin were designed from previously published sequences and used to amplify RNA isolated from either the E14 striatum or from EGF-generated spheres (Figure 4). Forty cycles of PCR detected message for BRK-1 in the embryonic striatum and in EGF-generated spheres. However, message for BMP-2 was detected only in the embryonic striatum and not in spheres. The specific identity of the BMP-2 and BRK-1 PCR products was confirmed by sequence analysis. The presence of BMP-2 and BRK-1 in the E14 striatum suggests possible roles in mouse CNS development.

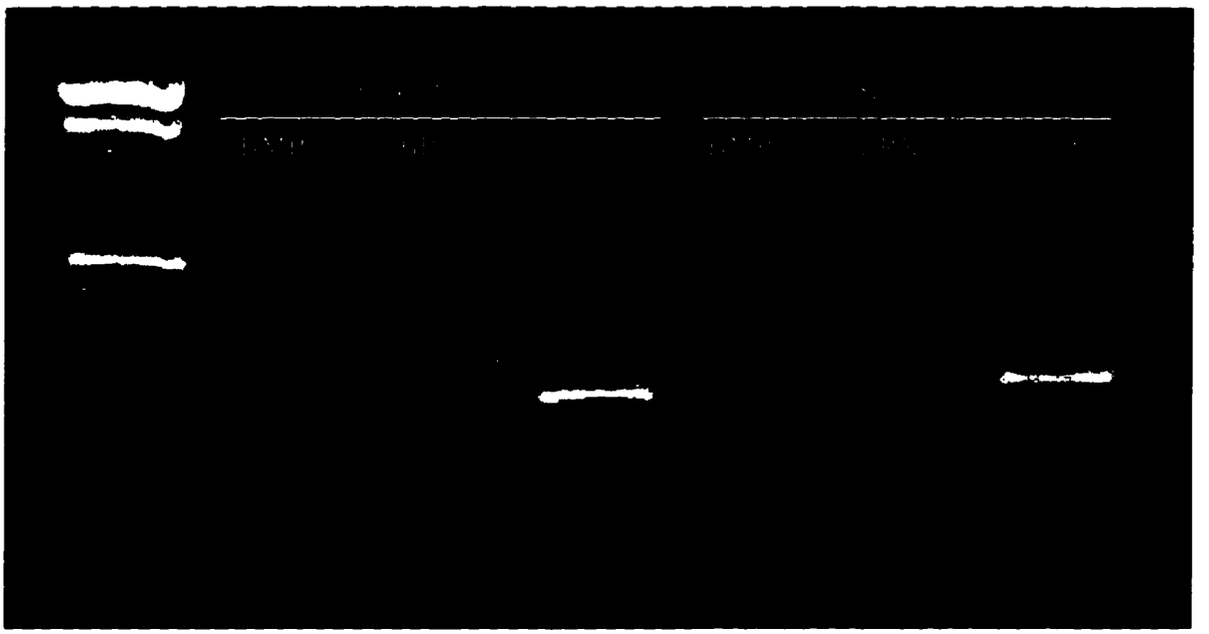
3.1.2 BMP-2 inhibits the proliferation of CNS stem cells in a dose-dependent manner

To investigate whether BMP-2 could influence the proliferation of CNS stem cells, the stem cell proliferation assay was used. This assay is based on the observation that all spheres generated in the presence of EGF are clonally-derived from one stem cell (Reynolds *et al.*, 1992; Reynolds and Weiss 1996). Therefore, the number of spheres that form in a single well of a 96-well plate is directly proportional to the number of stem cells initially plated. By using this assay, not only is it possible to monitor the number of spheres that form, but the size and morphology of spheres can also be

Figure 4:

BRK-1 transcripts are found in both the E14 striatum and in EGF-generated spheres whereas BMP-2 transcripts are detected only in the E14 striatum.

RT-PCR was used to probe for BMP-2 and BRK-1 (a type I receptor for BMP-2). Both BMP-2 and BRK-1 were present in the E14 striatum. BRK-1 message was detected in EGF-generated spheres while BMP-2 was not. Identification of the BRK-1 and BMP-2 PCR products was confirmed using sequence analysis.



assessed. To analyze the effect of an epigenetic factor on stem cell proliferation, all of these parameters can be compared to those of EGF-generated spheres (control conditions).

Cells isolated from the E14 mouse striatum were plated in 96-well plates in culture medium supplemented with EGF (20 ng/mL) in the absence or the presence of increasing concentrations of BMP-2. The average number of spheres generated was quantified after 10 DIV. In the absence of BMP-2, 34.0±3.8 spheres were produced per well (Figure 5). When low concentrations of BMP-2 (5 ng/mL) were present in the culture medium, a significant decrease (50%) in the number of spheres was observed (17.0±2.4; Figure 5). This decrease became more pronounced as the concentration of BMP-2 increased, with complete inhibition of stem cell proliferation occurring with approximately 30 ng/mL. In addition, the spheres that formed at concentrations between 5 and 30 ng/mL were no smaller than spheres grown in the absence of BMP-2 (data not shown). In the absence of EGF, BMP-2 did not stimulate sphere growth (data not shown). These results suggest that BMP-2 has a profound action on stem cell proliferation.

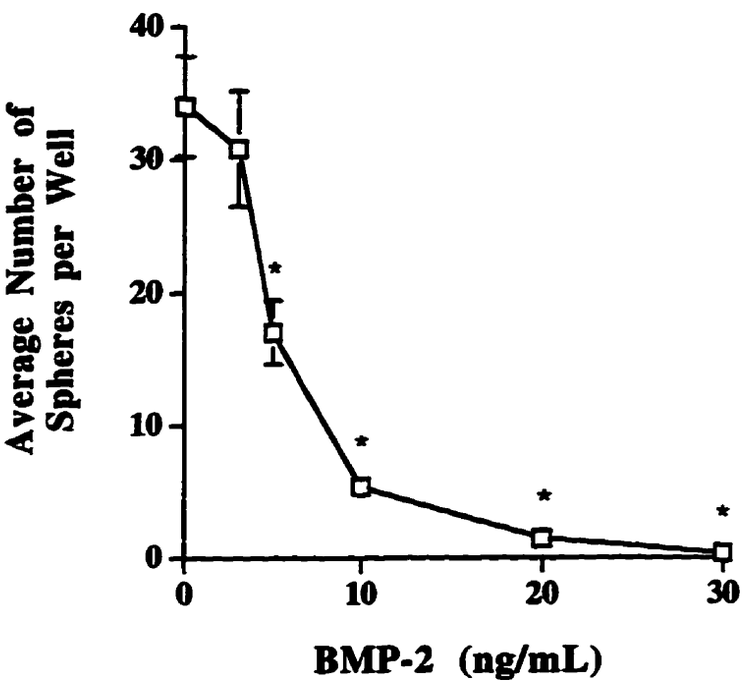
3.1.3 BMP-2 does not inhibit the proliferation of stem cell progeny

Since there are two populations of cells in EGF-generated spheres the next experiment was designed to test the effect of BMP-2 on stem cell progeny proliferation. This was accomplished using the rapid differentiation protocol (RDP). Unlike the stem cell proliferation assay that is used to characterize stem cell growth and proliferation, the RDP is used to observe the growth, differentiation and proliferation of stem cell progeny. A suspension of cells was plated onto cation-coated (poly-ornithine) coverslips that cause all of the cells to stick to the substrate and grow as a monolayer. Both the proliferation and differentiation of cells plated under these conditions can be monitored using immunocytochemical methods. Stem cells still proliferate under these conditions, yet they do so slowly and do not form spheres. Results from this

Figure 5:

BMP-2 inhibits the proliferation of CNS stem cells in a dose-dependent manner.

Cultures of E14 mouse striatum were plated in 96-well plates in culture medium supplemented with 20 ng/mL EGF in the absence or the presence of increasing concentrations of BMP-2. The average number of spheres per well was quantified after 10 DIV. As the concentration of BMP-2 increased, the average number of spheres produced per well decreased until complete inhibition of proliferation was reached with approximately 30 ng/mL BMP-2. All data points were compared to control (absence of BMP-2). * denotes data points significantly different than control ($p<0.05$; $n=3$).


Average Number of Spheres per Well
40
30
20
10
0
0
10
20
30
*
*
*
*
BMP-2 (ng/mL)

assay are assumed to be reflective of the stem cell progeny since they constitute the majority of the cells plated.

A suspension of dissociated pass-1 spheres was plated under RDP conditions on poly-ornithine coated coverslips in culture medium supplemented with EGF (20 ng/mL) in the absence or the presence of BMP-2 (30 ng/mL). After 7 DIV, cells were fixed for immunocytochemistry and counterstained with bis-benzamide to visualize nuclei. Under control conditions, the density of cells plated under these conditions typically increases 15-fold over 7 DIV (C. Wohl, unpublished observations). Therefore, if BMP-2 inhibits the proliferation of stem cell progeny, there should be a dramatic decrease in the number of cells present after 7 DIV. There was, in fact, no significant difference between the number of cells present per field in the absence (554±16) or the presence (523±14) of BMP-2 ($p>0.05$, $n=4$). These results indicate that BMP-2 does not affect the proliferation of stem cell progeny.

3.1.4 BMP-2 must be added immediately at plating to inhibit all sphere growth

One further experiment was designed to test the action of BMP-2 on stem cell progeny proliferation. For this experiment, the stem cell proliferation assay was used, but not in the same way that it was in section 3.1.2. This experiment was performed based on what is known about sphere composition. A mature (7-10 day old) sphere is comprised primarily of stem cell progeny (~80%). Assuming that the earliest proliferative events leading to sphere formation are likely dominated by stem cells, later sphere growth must be due to stem cell progeny proliferation. Therefore, if BMP-2 inhibits the proliferation of stem cell progeny, then the later that BMP-2 is added to cultures of stem cells, the larger the resultant spheres should become. To examine this possibility, the stem cell proliferation assay was used since the sizes of spheres are easily monitored.

BMP-2 (30 ng/mL) was added to each well of a 96-well plate either at plating, or its addition was delayed in 24 hour increments for up to 120 hours. The average number of spheres generated per well and their relative size was quantified after 10 DIV. BMP-2 had no effect on sphere size (data not shown) which is consistent with the results reported in section 3.1.2. Thus, BMP-2 does not inhibit the proliferation of stem cell progeny. However, as the addition of BMP-2 was delayed, the average number of spheres produced per well increased (Figure 6). If the addition of BMP-2 was delayed for 96 hours or longer, BMP-2 had no significant effect on the average number of spheres produced ($p>0.05$ for all timepoints greater than and including 96 hours; n=3, 8 wells/condition). The significance of this finding is discussed below.

3.1.5 BMP-2-induced inhibition of stem cell proliferation is reversible

One possible explanation for BMP-2 induced inhibition of stem cell proliferation is that it selectively kills the stem cells. A washout experiment was performed to test this possibility. If BMP-2 is an apoptotic or necrotic agent for stem cells then a brief (24-hour) exposure should be adequate to induce cellular death. Therefore, once BMP-2 is removed and replaced with EGF only, the appearance of spheres, or lack thereof, should indicate whether or not BMP-2 is killing stem cells. For this experiment, the stem cell proliferation assay was used as previously described.

Cells from the E14 striatum were plated in 96-well plates in culture medium supplemented with EGF (20 ng/mL) ± BMP-2 (3 ng/mL). After 24 hours, BMP-2 was removed and replaced with EGF-containing media only. The average number of spheres per well was quantified after 10 DIV and the results are illustrated in Figure 7. To serve as a control, there were two conditions where the medium was not changed after 24 hours. When BMP-2 was not in the culture medium, there were significantly more spheres present (50.2±3.8) than in cultures with BMP-2 (4.1±0.8). This result is in agreement with those reported in section 3.1.2 (Figure 5). When BMP-2 was removed

Figure 6:

BMP-2 must be added immediately at plating to inhibit all stem cell proliferation.

Cultures of E14 striata were plated in culture medium supplemented with EGF (20 ng/mL) in 96-well plates. The addition of BMP-2 (30 ng/mL) was delayed for 24, 48, 72, 96 or 120 hours after plating. The average number of spheres was quantified after 10 DIV. As the addition of BMP-2 is delayed, more spheres are gradually produced until control levels are attained (approximately 96 hours after plating). All data points were compared to control (absence of BMP-2). * denotes data points significantly different than control ($p<0.05$; $n=3$).

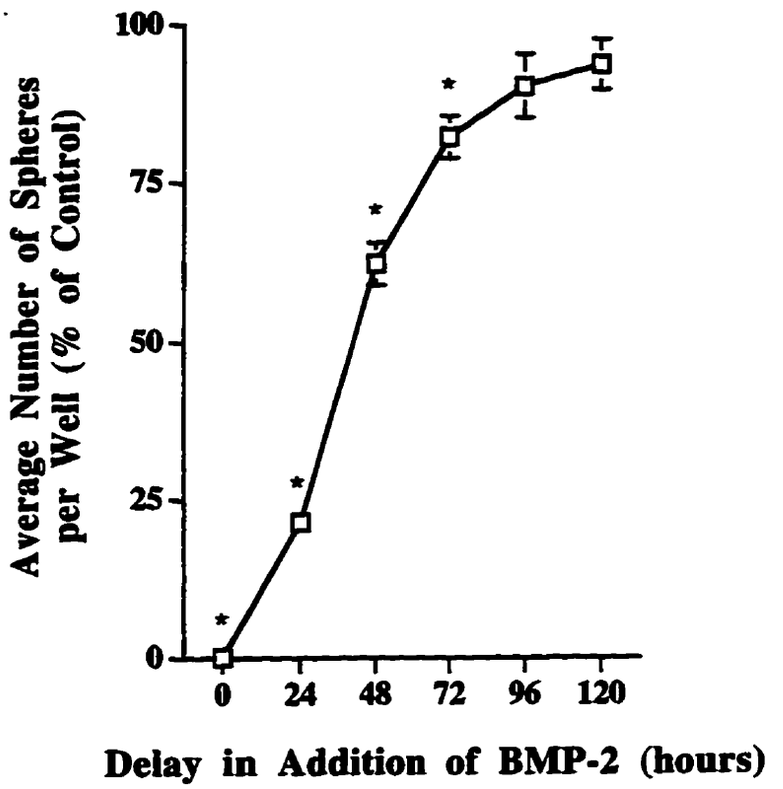
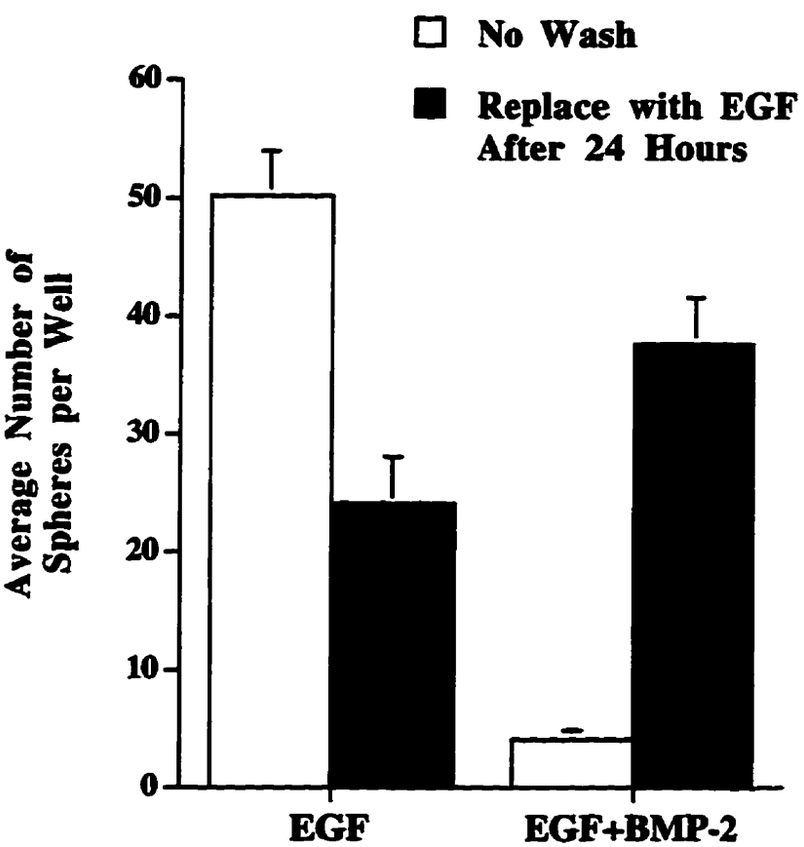

Average Number of Spheres per Well (% of Control)
100
75
50
25
0
0
24
48
72
96
120
Delay in Addition of BMP-2 (hours)

Figure 7:

BMP-2-induced inhibition of stem cell proliferation is reversible.

Cells from the E14 striata were plated in culture medium containing EGF (20 ng/mL) in the absence or the presence of BMP-2 (3 ng/mL). Twenty four hours after plating BMP-2 was removed and replaced with EGF only. Stem cells exposed to BMP-2 for 24 hours still retained their capacity to produce spheres (n=4).


No Wash
Replace with EGF
After 24 Hours
Average Number of
Spheres per Well
60
50
40
30
20
10
0
EGF
EGF+BMP-2

and replaced with EGF alone, there was a significant increase in the average number of spheres produced (37.7±3.9) compared to cultures that had been exposed to BMP-2 for the duration of the experiment (4.1±0.8). A full recovery (i.e. 50.2±3.8) was not expected since there was a significant decrease in the average number of spheres produced when EGF was removed and replaced with EGF alone (24.1±4.0). This decrease is due to the experimental design that results in the loss of some cells during the washing process. Unfortunately, I cannot explain why more spheres were produced in the presence (37.7±3.9) than in the absence (24.1±4.0) of BMP-2. Nonetheless, since stem cells exposed to BMP-2 could still produce spheres once BMP-2 was removed, the effect of BMP-2 on stem cell proliferation is reversible. Thus, the inhibition described in section 3.1.2 is likely not due to stem cell death.

3.2 DISCUSSION

As outlined in the introduction, BMPs are important molecules during embryogenesis. This, combined with the fact that embryonic CNS stem cells undoubtedly play a role during development, prompted me to test whether BMP-2 had an action on the biology of CNS stem cells. The results of this section demonstrate that BMP-2 can completely inhibit EGF-induced CNS stem cell proliferation. Moreover, this action does not extend to the proliferation of stem cell progeny. This is the first demonstration of a factor that can inhibit the proliferation of CNS stem cells.

Prior to studying the actions of BMP-2, I probed for both the ligand and receptor in the E14 striatum (the source of embryonic CNS stem cells) and in EGF-generated spheres (the product CNS stem cells). The results outlined in section 3.1.1 confirm the expression of BRK-1 (BMPR-IA) in both the E14 mouse striatum and EGF-generated spheres. Although there are currently three identified type I receptors for BMP-2; BRK-1 (BMPR-IA, Koenig, 1994), BMP-2R (BMPR-IB, Mishina, 1995) and ActR-I (ALK-3; ten Dijke *et al.*, 1994), BRK-1 was the only type I receptor we probed for. Whether the actions of BMP-2 on CNS stem cells are mediated through BRK-I remains to be determined. However, its presence in both the striatum and in spheres led me to hypothesize that BMP-2 could influence CNS stem cells.

The results of section 3.1.2 demonstrate that BMP-2 could completely block the EGF-induced proliferation of stem cells. In studies not reported here, I found that BMP-2 also inhibits the proliferation of passaged stem cells as well as stem cells isolated from the adult mouse (data not shown). The inhibitory action of BMP-2 on CNS stem cells is analogous to that of macrophage inflammatory protein 1α (MIP-1α), which inhibits both hematopoietic stem cell proliferation (Graham *et al.*, 1990; Lord *et al.*, 1993) and keratinocyte proliferation (Parkinson *et al.*, 1993). Similar to the action of BMP-2, the inhibition of hematopoietic stem cell proliferation by MIP-1α is

reversible (Graham *et al.*, 1990) and appears restricted to primitive precursor cells rather than cells committed to a specific lineage (Graham *et al.*, 1990). Parkinson *et al.* (1993) postulated that MIP-1α may be capable of inhibiting the proliferation of stem cells from many different tissues, however this is not the case. MIP-1α has no effect on the proliferation of intestinal crypt stem cells (Potten *et al.*, 1995) or CNS stem cells (C. Bjornson and S. Weiss, unpublished observations). Nonetheless, given MIP-1α's inhibition of proliferation of two different stem cells, studying the actions of BMP-2 on other tissues may be warranted.

BMP-2 induced inhibition of proliferation may not be surprising since other members of the TGF-ß superfamily have been characterized as reversible inhibitors of various cell types (Tucker *et al.*, 1984; Shipley *et al.*, 1986; Silberstein and Daniel, 1987; Russell *et al.*, 1988) including hematopoietic stem cells (Ohta *et al.*, 1987). Yet, some of these TGF-ß molecules can also promote apoptosis in blood (Taetle *et al.*, 1993; Selvakumaran *et al.*, 1994; Weller *et al.*, 1994), hepatic (Oberhammer *et al.*, 1991; Lin and Chou, 1992) and other cell types (Martikainen *et al.*, 1990; Rotello *et al.*, 1991; Yanagihara and Tsumuraya, 1992). Furthermore, BMPs have themselves been identified as molecules that mediate apoptosis in both the limb bud (Ganan *et al.*, 1996; Zou and Niswander, 1996) and the neural crest (Graham *et al.*, 1994). Therefore, to determine whether BMP-2 was a true inhibitor of proliferation or an apoptotic signal for stem cells, a washout experiment was performed (section 3.1.5, Figure 8). Since the removal of BMP-2 after 24 hours resulted in normal sphere growth, the effect is reversible and likely not apoptotic. Although one may expect an apoptotic signal to act very quickly (<24 hours), timepoints demonstrating that stem cells survive even after 72 hours of exposure may be more convincing than those described here.

There are many explanations that could account for BMP-2's inhibition of EGF-stimulated stem cell proliferation. BMP-2 could block the binding of EGF to its receptor by binding to the EGF receptor without activating it. This is the form of inhibition employed by the *Drosophila* protein ARGOS in blocking EGF signaling (Schweitzer *et al.*, 1995; Golembo *et al.*, 1996). BMP-2 could also bind directly to EGF itself, thereby inactivating it. Recent reports identify this as the mechanism that *noggin* (Re'em-Kalma *et al.*, 1995; Zimmerman *et al.*, 1996) and *chordin* (Sasai *et al.*, 1995; Piccolo *et al.*, 1996) use to inhibit BMP-4 from ventralizing presumptive dorsal mesoderm in *Xenopus*. Yet, the most plausible sites of interaction are intracellular. For example, EGF and BMP-2 signals could converge and have opposite effects on an intracellular effector molecule such as mitogen-activated protein kinase, the latter which is crucial to signaling by EGF (Hoshi *et al.*, 1988, 1989; Rossomando *et al.*, 1989; Gotoh *et al.*, 1990). Alternatively, BMP-2 may inhibit the activation of other intracellular molecules in a manner similar to that whereby TGF-ß downregulates the phosphorylation of various intracellular effector molecules required for insulin signaling (Atfi *et al.*, 1994).

Since there are at least two populations of cells within spheres, two experiments were performed to test whether BMP-2 could inhibit the proliferation of stem cell progeny as well. The first of these is outlined in section 3.1.3 where dissociated spheres were plated under RDP conditions in the presence of both EGF and BMP-2. If BMP-2 inhibited stem cell progeny proliferation, then the number of cells present after 7 DIV should decrease relative to those found with EGF alone. There was no statistical difference between the two conditions thereby suggesting that BMP-2 likely does not inhibit the proliferation of stem cell progeny. Still, one may have expected to observe a decrease in the number of cells since stem cells constitute 20% of a sphere. There are two likely explanations why this was not observed. First, stem cells proliferate much more slowly than stem cell progeny (Potten, 1986). Therefore, even under control conditions, stem cells would constitute much

less than 20% of the cellular population after 7 DIV. Secondly, if the proliferation of stem cell progeny is not affected by BMP-2, the abundance of stem cell progeny after 7 DIV would likely mask the lack of stem cell proliferation. Taken together, and by estimating that 50% of the stem cell progeny proliferate once per day, stem cells that initially represent 20% of the population would represent less than 2% after 7 DIV.

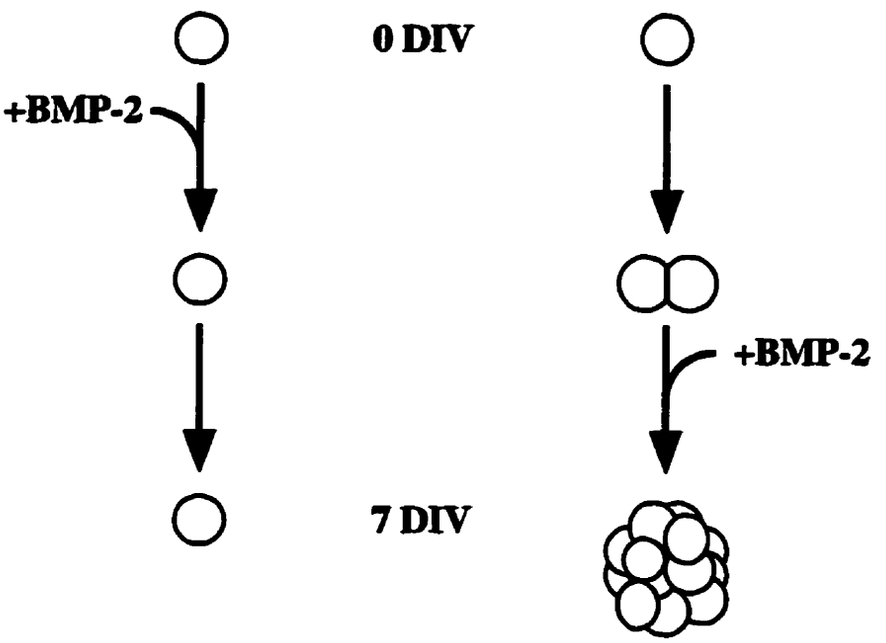
Another experiment designed to determine whether BMP-2 could inhibit stem cell progeny proliferation is outlined in section 3.1.4, where the addition of BMP-2 was delayed after plating. The foundation for this experiment is rooted in what we hypothesize about sphere growth. The earliest proliferative event leading to a sphere is by either a TSC and/or a MPC and the later expansion is likely dominated by stem cell progeny since the latter constitute 80% of a sphere. Therefore, if the addition of BMP-2 is delayed, and it possesses the capability to inhibit stem cell progeny inhibition, the sizes of the spheres produced should be smaller the earlier BMP-2 is added to the culture (as assayed after 10 DIV). There were two observations made from these experiments: 1) spheres that formed in the presence of BMP-2 were no smaller than control spheres, and 2) complete inhibition of stem cell proliferation occurred only if BMP-2 was added immediately at plating. Not only do these data support the observation that BMP-2 does not inhibit stem cell progeny proliferation (section 3.1.2), they raise a very interesting possibility that may further define the actions of BMP-2 on stem cell proliferation.

The data presented in Figure 7 may be transformed to fit a normal distribution with a mean of approximately 48 hours. This may define two characteristics of stem cell proliferation: 1) the temporal control of proliferation of individual stem cells is asynchronous, and 2) this asynchrony may represent the cell cycle time for CNS stem cells. The latter is based on the assumption that BMP-2 can only inhibit stem cell proliferation before the first cellular division has occurred (Figure 8), which may be inferred from the

Figure 8:

Schematic representation of the proposed mechanism by which BMP-2 inhibits CNS stem cell proliferation.

The results of the delayed addition experiment (section 3.1.4) indicate that EGF-responsive stem cell growth is not affected if BMP-2 is added 96 hours after plating. Furthermore, spheres produced in the presence of BMP-2 are no smaller than control spheres. Taken together, these results may indicate that BMP-2 can inhibit CNS stem cell proliferation, but only if it is added before the first stem cell division.


0 DIV
+BMP-2
+BMP-2
7 DIV

results found in section 3.1.4. From this, I propose that the apparent cell cycle time for a CNS stem cell is in the range of approximately 100 hours. Hematopoietic stem cells have an average cell cycle time of about 120 hours and skin epithelia stem cells have a cycle of about 125 hours (Potten, 1986). Therefore, a 100 hour cell cycle time is in the range of previously reported cell cycle times for stem cells of other systems. To further characterize the actions of BMP-2 on stem cell proliferation, and to test the accuracy of the cell cycle time reported here, a clonal analysis should be performed. From this, the precise cell cycle times for CNS stem cells could be measured and the ability of BMP-2 to inhibit proliferation before and/or after the first division can be examined.

4 BMP- 2 ENHANCES THE DIFFERENTIATION OF NEURONS FROM EGF-GENERATED STEM CELL PROGENY

4.1 RESULTS

4.1.1 BMP-2 increases the number of astrocytes and neurons produced from stem cell progeny

In section 3, BMP-2 was identified as an inhibitor of stem cell proliferation, yet it had no effect on the proliferation of stem cell progeny. In this section I ask whether BMP-2 influences the differentiation of stem cell progeny. To address this, stem cell progeny were cultured under RDP conditions. In all of the following experiments, a suspension of dissociated pass-1 EGF-generated spheres was plated on poly-ornithine coated coverslips in culture medium with or without BMP-2 (please note that no fetal calf serum or EGF is used in any of these experiments). Cells were fixed for indirect immunocytochemistry after 7 DIV. Antibodies and/or antisera specific for various cellular antigens were used to identify the differentiated phenotypes, while the nuclear stain bis-benzamide was used to quantify the total number of cells. To quantify the results of each experiment, 20 independent, non-overlapping fields were counted per coverslip with a minimum of 2 coverslips per experiment.

In the first experiment, the presence of all three major cell types found in stem cell progeny RDPs - astrocytes, oligodendrocytes and neurons - was examined in the absence or presence of BMP-2 using immunocytochemical methods. Neurons were identified using an antibody directed against the neuronal specific antigen type III ß-tubulin (Geisert and Frankfurter, 1989; Reynolds and Weiss, 1996). The number of ß-tubulin immunoreactive cells present in cultures treated with BMP-2 (50 ng/mL) increased approximately 14-fold over those seen in control (Table 1; $p<0.05$, $n=3$). This result was confirmed with an antibody directed against another neuron specific antigen,

Table 1: The relative abundance of differentiated neural cell types in stem cell progeny RDP cultures treated with BMP-2.

Phenotype	Relative Abundance (% total cells)		Fold Increase	
	Control	+BMP-2		
Neurons	0.07±0.02	0.98±0.06	14.6	**
Astrocytes	0.74±0.07	5.90±0.32	8.0	**
Oligodendrocytes	5.80±0.28	6.41±0.26	1.1	

** indicates a significant increase in cellular populations compared to control ($p<0.05$)

microtubule associated protein-2 (data not shown). Astrocytes were identified with the astrocyte-specific marker, glial fibrillary acidic protein (GFAP). The presence of BMP-2 in the culture medium induced an 8-fold increase in the number of astrocytes produced (Table 1; $p<0.05$, $n=4$). The number of oligodendrocytes, however, was not affected by BMP-2 in the culture medium. Using a monoclonal antibody directed against the oligodendrocyte specific antigen O4, there was no significant increase or decrease in the number of oligodendrocytes produced in the presence of BMP-2 (Table 1; $p>0.05$, $n=3$). As the number of cells present in all of these cultures was expressed as a percentage of total cells, it was necessary to confirm that the same number of total cells was present in the absence or presence of BMP-2. Indeed, the same number of cells was present in the absence (187 ± 7) and the presence (203 ± 10) of BMP-2 after 7 DIV ($p>0.05$, $n=3$). This result validates my use of percentages to assess cellular numbers.

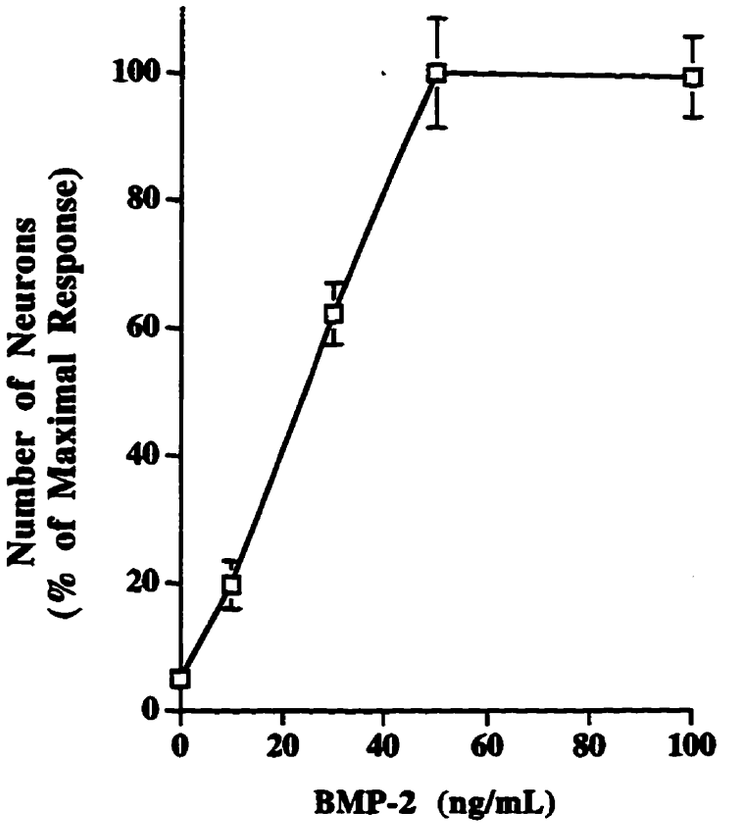
Since there was no change in the number of oligodendrocytes produced in the presence of BMP-2, and the increase in astrocyte numbers has been previously described (Mehler *et al.*, 1995; Gross *et al.*, 1996), all of the following experiments were designed to characterize the BMP-2 induced increase in the production of neurons by stem cell progeny. The increase in neuron-like cells occurred in a dose-dependent manner with an apparent saturation of 50 ng/mL (Figure 9). Therefore, all subsequent experiments were performed using this concentration of BMP-2.

The experiments described in sections 4.1.2 to 4.1.4 were performed to establish the mechanism/process by which BMP-2 increases neuronal numbers. These three mechanisms/processes are described in the introduction (section 1.4) and illustrated schematically in Figure 3.

Figure 9:

BMP-2 increases the production of neurons by stem cell progeny in a dose dependent manner.

EGF-generated spheres were dissociated and plated onto poly-ornithine coated coverslips in defined culture medium in the absence or presence of BMP-2. ß-tubulin immunoreactive cell numbers increase as the dose of BMP-2 increases with an apparent saturation with approximately 50 ng/mL BMP-2. The data is represented as a percentage of maximal response to account for experimental variability (n=3).


Number of Neurons
(% of Maximal Response)
100
80
60
40
20
0
0
20
40
60
80
100
BMP-2 (ng/mL)

4.1.2 BMP-2 is not a proliferative factor for neuronal precursor cells

The following experiment was performed to determine if BMP-2 is mitogenic for neuronal precursors. To detect mitotic activity in cells plated under RDP conditions, the thymidine analogue BrdU was used. BrdU is incorporated into the DNA of mitotically active cells and can be detected immunocytochemically using a monoclonal antibody (Granzber, 1982). Therefore, if BMP-2 is acting as a mitogen for neuronal precursor cells, it can be monitored using double-label immunocytochemistry for ß-tubulin and BrdU. Although this issue was partially addressed in section 4.1.1, an increase in neuronal precursor proliferation may occur without a detectable increase in total cells since the neuronal population constitutes less than 1% of the overall population present after 7 DIV (Table 1).

Cells from dissociated EGF-generated spheres were plated under RDP conditions and supplemented with 1 µM BrdU. The results of two independent experiments are illustrated in Figure 10. Of the 15 neurons produced in the absence of BMP-2, only one incorporated BrdU. This is consistent with the idea that cells are somewhat mitotically quiescent under these conditions. If BMP-2 is a proliferative factor for neuronal precursor cells, all of the newly-generated neurons should incorporate BrdU (Figure 10). However, in the presence of BMP-2, none of the 85 total identified neurons incorporated BrdU, suggesting that BMP-2 is not a proliferative factor for neuronal precursor cells (Figure 10; n=2).

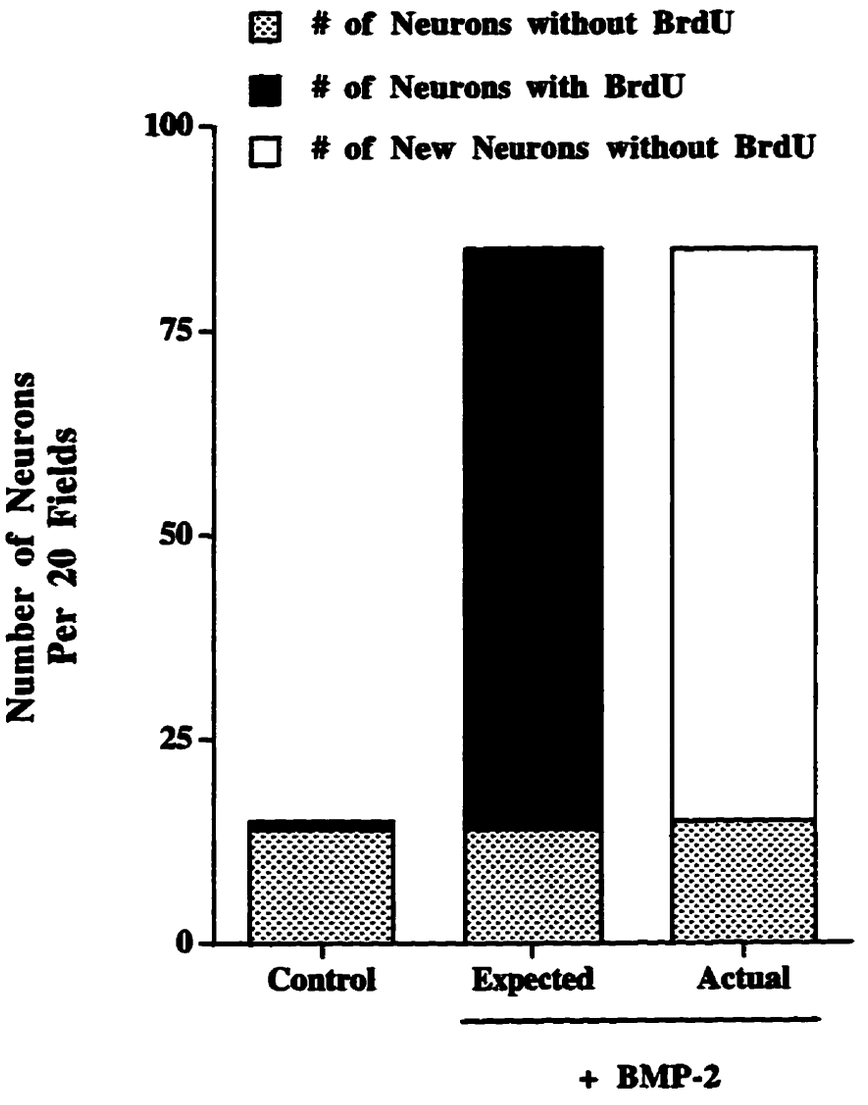
4.1.3 BMP-2 is not a survival factor for neurons or neuronal precursor cells

Another mechanism/process that BMP-2 could use to increase neuronal numbers is by enhancing their survival. Based on the definition (section 1.4.2) that survival factors need to be present continuously to exert their effect, two experiments were performed. The addition of BMP-2 was either delayed after initial plating, or it was added and then removed. Since

Figure 10:

BMP-2 is not a proliferative factor for neuronal precursor cells.

Dissociated EGF-generated spheres were cultured in medium containing 1 μM BrdU, in the absence or the presence of BMP-2. Under control conditions, very few neurons were produced with very little BrdU incorporation. If BMP-2 is a proliferative factor for neuronal precursor cells, the expected result is that most or all of the newly generated neurons would be immunoreactive for BrdU. Since the actual result was a complete lack of BrdU immunoreactive neurons, BMP-2 is likely not a proliferative factor for neuronal precursor cells (n=2).


# of Neurons without BrdU
# of Neurons with BrdU
# of New Neurons without BrdU
Number of Neurons Per 20 Fields
100
75
50
25
0
Control
Expected
Actual
+ BMP-2

BMP-2 would not be continuously present under these conditions, there should be a decrease in the number of neurons produced.

In the first experiment, the addition of BMP-2 was delayed after the cells were initially plated. Cells were plated under RDP conditions and the addition of BMP-2 was delayed 1, 2, 3, 4, 5, or 6 days after plating. All cultures were assayed for the presence of neurons 7 DIV after initial plating, regardless of exposure time to BMP-2. As illustrated in Figure 11A, the addition of BMP-2 could be delayed for up to 4 days without significantly decreasing the number of neurons seen after 7 DIV ($p<0.05$ for all data points compared to control, n=3). There was a significant decrease in the number of neurons produced if the addition of BMP-2 was delayed for 5 or 6 days ($p<0.05$ compared to control, n=3). From these data, it appears that stem cell progeny can produce the same number of neurons even if the addition of BMP-2 is delayed for 4 days after plating.

The second experiment tested to what extent BMP-2-induced increases in neuronal numbers would persist when BMP-2 was added at plating and then removed at varying intervals thereafter. Dissociated EGF-generated spheres were plated under RDP conditions. BMP-2 was added immediately at plating then it was removed by washing 4 times with culture medium at various timepoints. Very brief exposure of cultures to BMP-2 induced an increase in neuronal numbers (Figure 11B). Moreover, the data suggests that only a 48-hour exposure to BMP-2 is required for an increase in neuronal numbers comparable to continuous (7 DIV) growth factor presence (n=4). Taken together, these experiments demonstrate that the continual presence of BMP-2 is not required for its induced increase in the number of neurons from stem cell progeny. Therefore, according to my definition, BMP-2 is not a survival factor for neurons.

Figure 11:

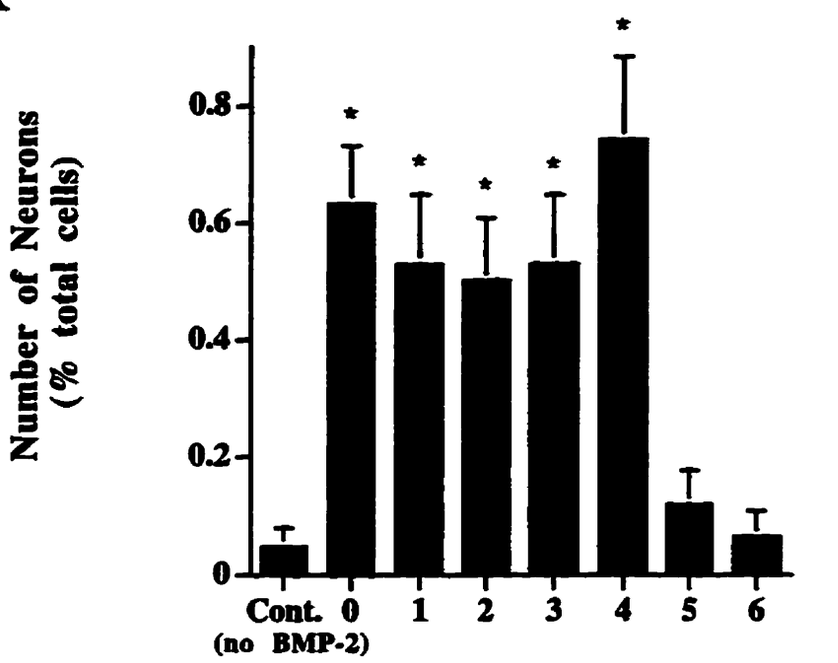
BMP-2 is not a survival factor for neurons or neuronal precursor cells.

Cultures of dissociated EGF-generated spheres were plated on poly-ornithine coated coverslips in culture medium.

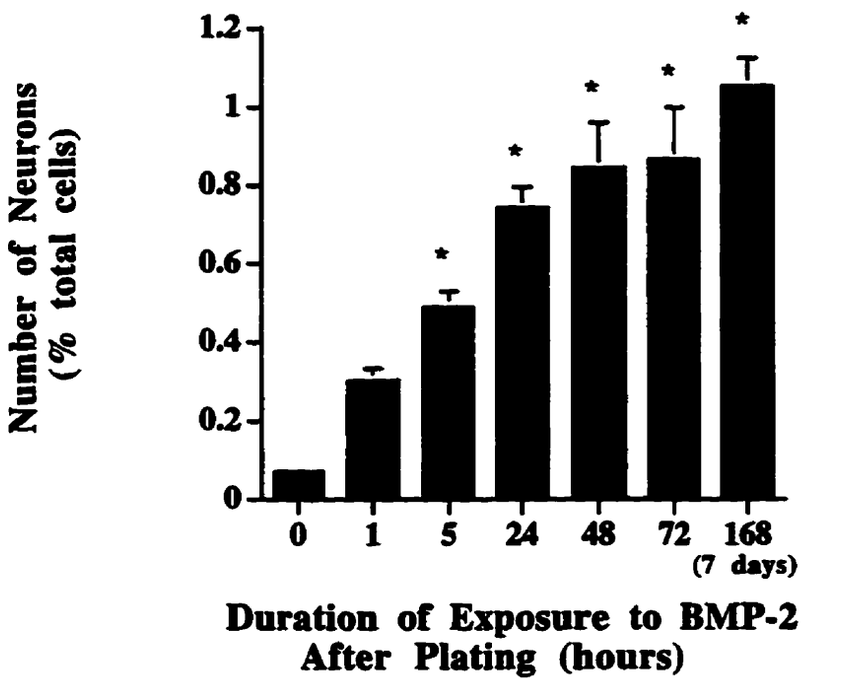
(A) The addition of BMP-2 was delayed either 1, 2, 3, 4, 5, or 6 days after plating. All data points were compared to control (absence of BMP-2). * denotes data points significantly different than control ($p<0.05$; $n=3$).

(B) BMP-2 was added immediately at plating and then removed at different timepoints. All data points were compared to control (absence of BMP-2). * denotes data points significantly different than control ($p<0.05$; $n=4$).

A


Number of Neurons
(% total cells)
0.8
0.6
0.4
0.2
0
Cont. 0 1 2 3 4 5 6
(no BMP-2)
Delay in Addition of BMP-2 (days)

B


Number of Neurons
(% total cells)
1.2
1
0.8
0.6
0.4
0.2
0
0 1 5 24 48 72 168
(7 days)
Duration of Exposure to BMP-2
After Plating (hours)

4.1.4 BMP-2 acts on cells that would have otherwise remained undifferentiated or become non-neuronal

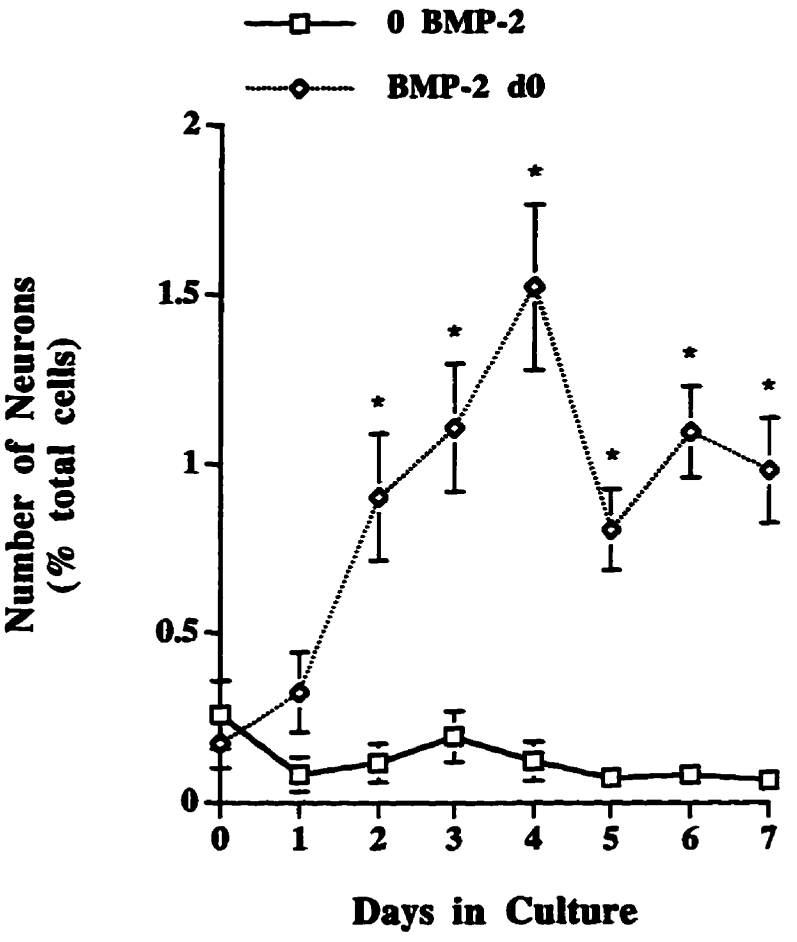
The results of the previous experiments suggest that BMP-2 is neither a proliferative nor a survival factor for neurons. Therefore, BMP-2 is likely a differentiation factor for neurons and/or neuronal precursors. According to my definition (section 1.4.3), if BMP-2 is a neuronal differentiation factor, one would expect a gradual increase in the number of neurons over the 7 day culture period. To test this, the appearance of neurons over time was examined both in the absence and the presence of BMP-2.

Dissociated pass-1 EGF-generated spheres were plated under RDP conditions in the absence or the presence of BMP-2 (50 ng/mL). Every 24 hours, coverslips were fixed and processed for immunocytochemistry (Figure 12). There was no significant difference between the number of neurons present in the absence (0.26±0.10%) or the presence (0.18±0.07%) of BMP-2 on day 0 ($p<0.05$, $n=3$). However, there is a significant increase in the number of neurons after 2 DIV in the presence of BMP-2 (0.90±0.13%, $p<0.05$). This increase continues until day 4 when the maximal number of neurons is detected (1.52±0.17%). The number of neurons appear to diminish over the last three days in culture until levels reported in section 4.1.1 are observed (0.98±0.06%). In the absence of BMP-2, the number of neurons remains relatively constant over the 7-day culture period (Figure 12). These data are consistent with my prediction of a gradual increase in neuronal numbers over time, if BMP-2 is acting as a differentiation factor for neurons.

Figure 12:

BMP-2 acts on cells that would have otherwise remained undifferentiated or become non-neuronal.

Cultures of dissociated EGF-generated spheres were plated on poly-ornithine coated coverslips in the presence of culture medium both in the absence or the presence of BMP-2. All data points were compared to control (percentage of neurons at day 0±BMP-2). * denotes data points significantly different than control ($p<0.05$; $n=3$).


0 BMP-2
BMP-2 d0
Number of Neurons
(% total cells)
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
Days in Culture

4.1.5 Neurons produced in the presence of BMP-2 exhibit longer neurites and are immunoreactive for GABA

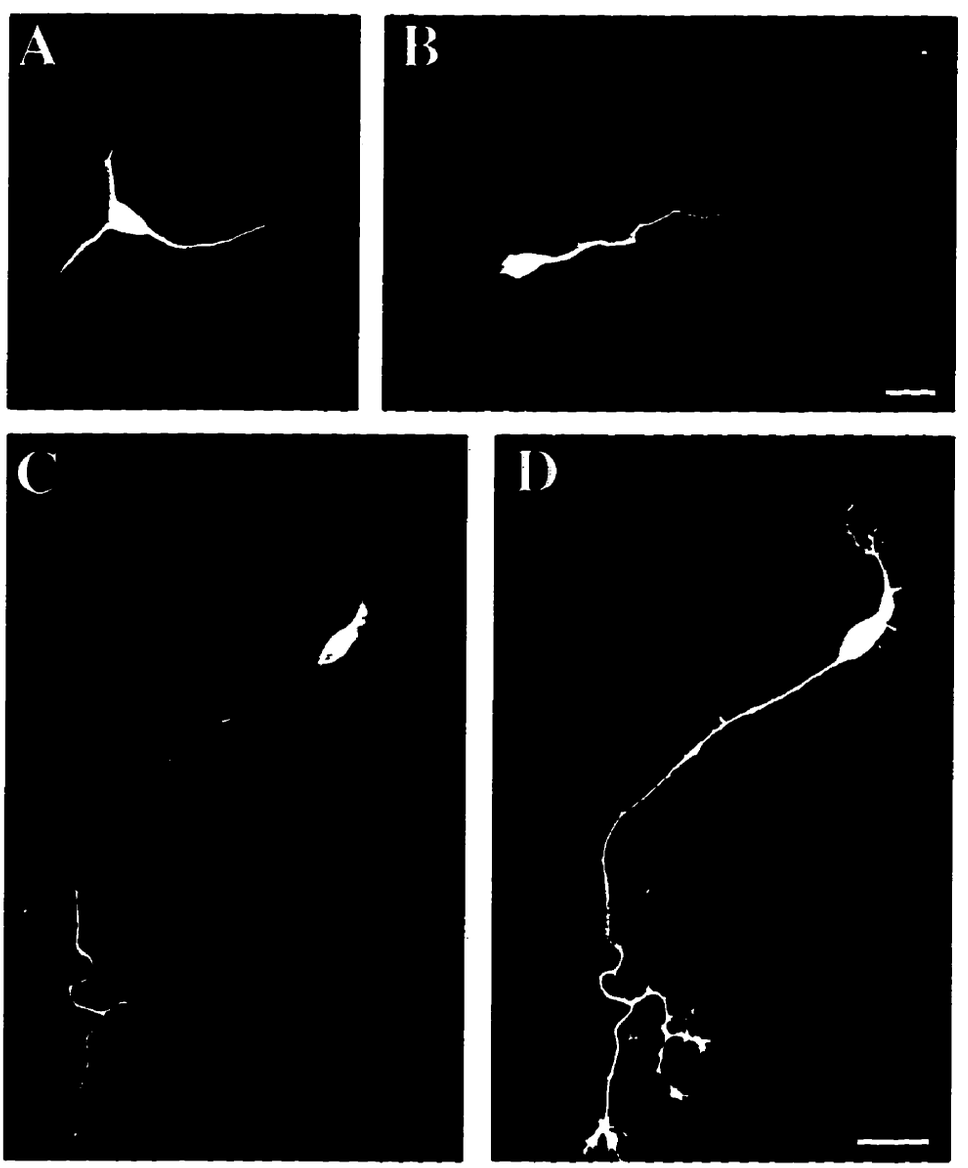
As the results of the previous section indicate that BMP-2 is a differentiation factor for neurons, I next asked whether BMP-2 influenced the morphology or the neurotransmitter phenotype of the neurons produced. Neurons grown in the presence of BMP-2, identified with a ß-tubulin antibody, exhibited longer neurites (Figure 13B, C) than those in cultures without BMP-2 (Figure 13A). Unlike a previous report that indicated enhanced outgrowth *and* branching of neuritic processes in the presence of BDNF (Ahmed *et al.*, 1995), BMP-2 appears to enhance process outgrowth without an action on process branching.

To determine whether BMP-2 promotes the production of a neuronal phenotype not seen under control conditions, indirect immunocytochemistry was performed. Virtually all ß-tubulin immunoreactive neurons generated in the absence (95.8±4.2%) or the presence (92.7±3.0%) of BMP-2 stained positively with GABA antisera (Fig. 13C, D; $p>0.05$, $n=3$). This result is consistent with previous reports that identified GABA as the principal neurotransmitter expressed by neurons generated by EGF-responsive CNS stem cell progeny (Reynolds *et al.*, 1992; Ahmed *et al.*, 1995). Thus, BMP-2 does not appear to influence the neurotransmitter phenotype of these neurons.

Figure 13:

Neurons produced in the presence of BMP-2 have longer neurites and are immunoreactive for GABA.

Indirect immunocytochemistry was performed on stem cell progeny cultured in the absence or the presence of BMP-2 for 7 DIV. (A) ß-tubulin immunoreactivity revealed that neurons not exposed to BMP-2 had very short neurites whereas neurons exposed to BMP-2 (B) had much longer neurites (A and B to the same scale; calibration bar, 30 μm). (C) ß-tubulin immunoreactive cells produced in the presence of BMP-2 were principally GABAergic (D) (C and D to the same scale; calibration bar, 30 μm).


A
B
C
D

4.2 DISCUSSION

In this section of the thesis, I asked what action, if any, BMP-2 may have on the differentiation of stem cell progeny. My results indicate that BMP-2 is capable of enhancing the differentiation of astrocytes and neurons from stem cell progeny. The increase in neuronal numbers appears to be due to BMP-2 acting as a differentiation factor, not as a survival or proliferative factor. Qualitatively, BMP-2 also appears to enhance neurite outgrowth. Therefore, these data suggest that BMP-2 is a potent neuronal differentiation factor for stem cell progeny.

There have been two recent reports of the actions of BMP-2 on stem cells isolated from the PNS and CNS. Each of these suggest a different role for BMP-2 depending on the system analyzed. PNS stem cells are similar to CNS stem cells in many respects. PNS stem cells proliferate, are capable of self maintenance and they produce the principal cell types of the neural crest (Stemple and Anderson, 1992). When PNS stem cells are cultured at clonal density in minimal growth medium, they will proliferate to form a cluster of cells, the majority of which are likely PNS MPCs (Stemple and Anderson, 1992). These PNS MPCs will produce the major cell types found in the neural crest - neurons, glia, and smooth muscle (Stemple and Anderson, 1992; Shah *et al.*, 1994, 1996). There is a great deal of interest in the control of PNS stem cells since similar mechanisms may regulate CNS stem cell biology. The control of the differentiation of PNS stem cell progeny has recently been addressed (Shah *et al.* 1994, 1996). In these two reports, three epigenetic factors radically changed the differentiated phenotypes produced by PNS stem cell progeny (Shah *et al.* 1994, 1996). For example, in the presence of GGF, all of the PNS stem cell progeny generated were glial (Shah *et al.*, 1994, 1996). Not only were neurons completely absent in the presence of GGF, careful analysis of these cultures demonstrated that the neuronal *lineage* was never present (Shah *et al.*, 1994). Therefore, the conclusion drawn from these studies was that GGF is a glial determination factor for PNS stem cell progeny (Shah *et al.*,

1994, 1996). A determination factor influences the fate of an uncommitted cell while a differentiation factor will advance the maturation state of a committed cell (Potten and Loeffler, 1990; Alberts *et al.*, 1994). Furthermore, GGF acts instructively rather than passively, since glia appeared at the expense of the other cell types, i.e. the production of the other phenotypes was suppressed (Shah *et al.*, 1994, 1996). GGF is not the only determination factor for PNS stem cell progeny. BMP-2 is a neuronal determination factor and TGF-ß1 is a determination factor for smooth muscle cells (Shah *et al.*, 1996). Though the action of BMP-2 was only 80% as effective as the other factors (Shah *et al.*, 1996), this was attributed to the cross-reactivity that could occur between BMP-2 and TGF-ß1 receptors (members of the same superfamily). In support of this contention was the observation the only phenotype that contaminated the neuronal clusters was smooth muscle (Shah *et al.*, 1996).

Though BMP-2 enhances the production of neurons from both CNS and PNS stem cells, these two actions are very different. First, the factors used by Shah *et al.* (1994, 1996) were added immediately at plating. This insured that all cells would be exposed to the different factors as soon as they were produced. This strategy would not work for CNS stem cells however. As outlined in the first section of this thesis, if BMP-2 is added immediately at plating there is an inhibition of stem cell proliferation. Therefore, the action of BMP-2 on CNS stem cells is to inhibit their proliferation, not to influence their fate. Second, since BMP-2 induced an almost complete phenotypic switch (~80%) in the PNS at the expense of other cell types (Shah *et al.*, 1996), it is considered a determination factor rather than a differentiation factor. My experiments suggest that BMP-2 enhances the differentiation of neurons from stem cell progeny and the neuronal increase observed does not come at the expense of another cell type. But, not all cells in my experiments become differentiated into a detectable phenotype, therefore I cannot conclude that the increase in neuronal numbers did not occur at the expense of another cell type. Although my experiments indicate that BMP-2 is a differentiation

factor, once the survival/differentiation rate of CNS stem cell progeny has been improved, it may be further characterized as a determination factor.

Recently, a study of the actions of BMP-2 on the differentiation of EGF-generated CNS stem cell progeny was reported (Mehler *et al.*, 1995; Gross *et al.*, 1996). In these studies, BMP-2 appeared to enhance the production of astrocytes from CNS stem cell progeny (Mehler *et al.*, 1995; Gross *et al.*, 1996). Surprisingly, the increase in astrocyte numbers was found to be accompanied by a decrease in the number of neurons and oligodendrocytes (Gross *et al.*, 1996). The authors conclude that the actions of BMP-2 on stem cell progeny is similar to the effect of GGF on PNS stem cell progeny (Shah *et al.*, 1994). Since the differentiation of astrocytes occurred at the expense of all the other cell types, it was concluded that BMP-2 is a glial determination factor for CNS stem cell progeny (Gross *et al.*, 1996). The results of my work partially support the observation of Gross *et al.* (1996). I too see an increase in the number of astrocytes in the presence of BMP-2, however I observe an increase in the number of neurons produced and no change in oligodendrocyte numbers (Table 1). This discrepancy may be somewhat difficult to resolve since Gross *et al.* (1996) and I used similar, but not identical, culture preparations. There are a few differences between the two studies, however. The primary source of the CNS stem cells in the Gross *et al.* (1996) study was E17, not E14, and the spheres that were used were passaged 3 or 4 times instead of once. Although neither of these differences should account for the difference between the two studies, one other may. While I attempted to keep all of the culture conditions constant when the effect of BMP-2 on astrocyte, neuron and oligodendrocyte numbers was quantified, Gross *et al.* (1996) did not. For example, the substrate for neuronal growth was laminin (Gross *et al.*, 1996) instead of poly-ornithine. This may explain the difference in neuronal numbers by virtue of the cell adhesion molecules upregulated by BMP-2. It is possible that the production of L1 and NCAM (Paralkar *et al.*, 1992; Perides *et*

al., 1994), combined with laminin, altered the differentiation, survival or proliferation of neurons and/or neuronal precursor cells in these cultures.

The experiments outlined in sections 4.1.2 to 4.1.4 were performed in an attempt to identify the mechanism/process used by BMP-2 to increase neuronal numbers. From these data, it is plausible to suggest that BMP-2 induces the *differentiation* of neurons from stem cell progeny. The working definitions of proliferation, survival and differentiation outlined in the introduction (section 1.4) form the basis for this conclusion. Though these processes occur in the developing animal, it is unlikely that they operate in a mutually exclusive fashion. For example, it is very likely that differentiation could occur as a result of cellular survival, or conversely the survival of a cell could be dependent on its differentiation. These two phenomenon are not trivial to dissect from one another, though others have noted that proliferation and differentiation can, and often do, operate independently of one another (Roher and Thoenen, 1987; LoPresti *et al.*, 1992). Nonetheless, there is undoubtedly some accuracy to these definitions, which were included to simplify our situation and allow for clearer interpretation of our data.

Earlier work from our lab demonstrated that BDNF is a differentiation factor for neurons and a potent inducer of neurite extension (Ahmed *et al.*, 1995). BMP-2 also appears to be a differentiation factor for neurons that enhances neurite outgrowth. Like BMP-2, BDNF did not increase neuronal numbers through enhanced proliferation or survival of neurons (Ahmed *et al.*, 1995). Furthermore, neither BMP-2 nor BDNF conferred neurotransmitter specificity to neurons (Ahmed *et al.*, 1995). The difference between the two studies is probably where each molecule is likely acting in the neuronal differentiation process. If BDNF is removed after 48 hours, and the neuronal numbers are assayed after 10 DIV, BDNF has no effect on the number of neurons produced (Ahmed *et al.*, 1995). However, if the addition of BDNF is delayed for 10 DIV, and the neuronal numbers are assayed after 21 DIV, there is an increase in the number of neurons. From this, it appears that

cells likely had to differentiate, or mature, to a point where they would become susceptible to the actions of BDNF. On the other hand, a 48-hour exposure of stem cell progeny to BMP-2 is sufficient for a complete increase in the number of neurons produced (Figure 11B). Furthermore, if the addition of BMP-2 is delayed longer than 4 DIV, there is no increase in the number of neurons produced (Figure 11A). These results combined with those found for BDNF suggest that cells are susceptible to the actions of BMP-2 earlier than BDNF. This sequential susceptibility to different factors during neuronal development has been documented before. As PNS neurons differentiate they become responsive to the effects of one factor while losing their susceptibility to another (Vogel and Davies, 1991; Buchman and Davies, 1993; Davies *et al.*, 1993; for review see Davies, 1994). Therefore, although BMP-2 and BDNF have very similar effects on the differentiation of stem cell progeny, there are subtle differences that set them apart from each other.

5 GENERAL CONCLUSIONS

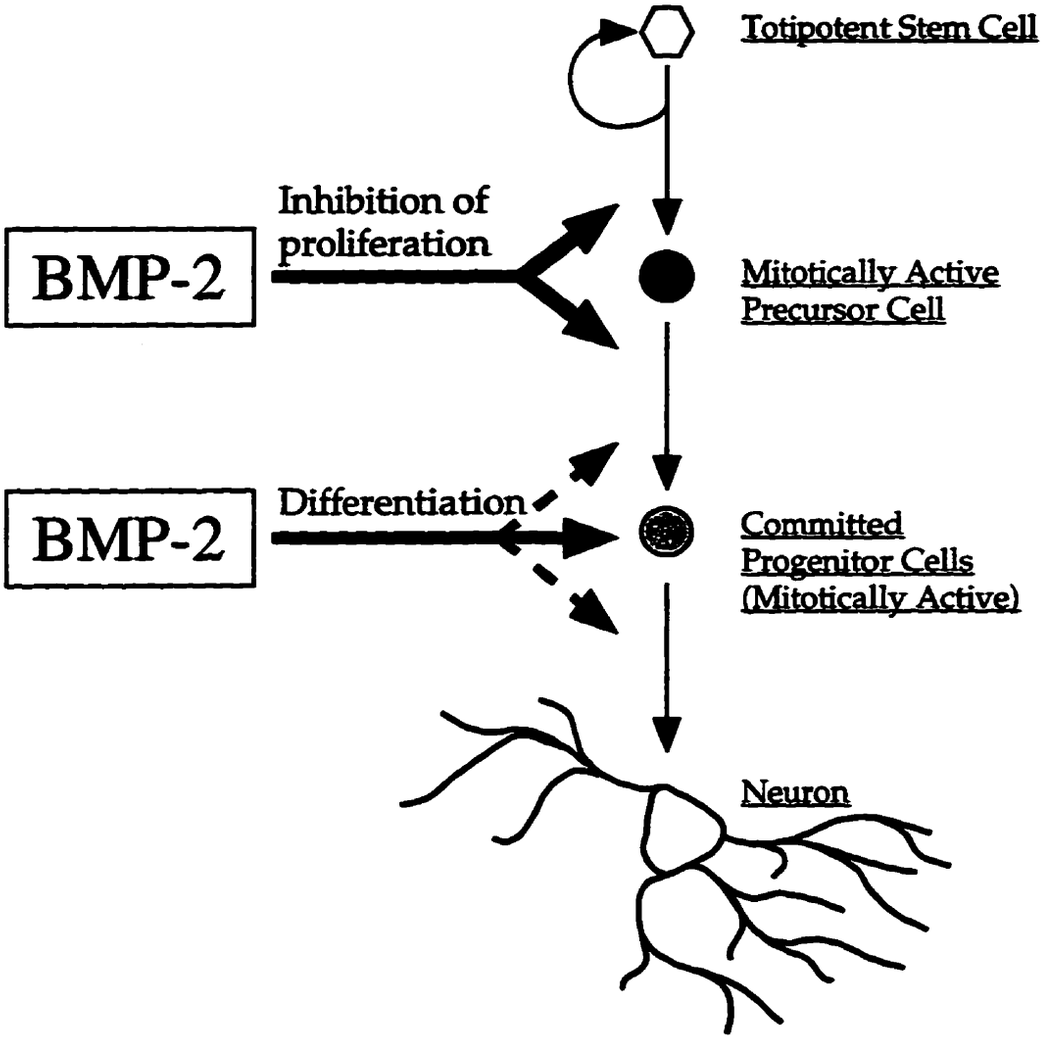
There is now compelling evidence that the multipotent cell isolated from the embryonic (Reynolds *et al.*, 1992) and the adult (Reynolds and Weiss, 1992) mouse striatum is indeed a stem cell (Reynolds and Weiss, 1996; Weiss *et al.*, 1996). Therefore, it seems likely that CNS stem cells would be governed by a similar hierarchy as that found in neuropoiesis and hematopoiesis. At the top of the hierarchy there is a TSC, then an MPC, followed by a committed progenitor cell and finally a differentiated cell (Figure 3). Similar to the hematopoietic and neuropoietic systems, the isolation of a CNS stem cell that can be easily manipulated *in vitro* presents a very attractive model to use in the discovery of signals that determine cell fate. These stem cell hierarchies are controlled by the presence of various extrinsic factors. These factors can influence the proliferation, the survival and/or the differentiation of the various cells. The results of this study describe the inhibition of CNS stem cell proliferation and the enhancement of neuronal numbers through enhanced differentiation, not proliferation or survival, from stem cell progeny in the presence of BMP-2.

So, where in the CNS stem cell hierarchy does BMP-2 fit? Since it is hypothesized that both MPCs and TSCs are capable of producing spheres, the proliferation of both is likely inhibited by the action of BMP-2 (Figure 14). Yet, it is still possible that BMP-2 could inhibit the proliferation of *either* the MPC or the TSC. The most effective way to address this is to separate TSCs and MPCs using specific markers similar to those found in the hematopoietic field (Civin *et al.*, 1984; Thoma *et al.*, 1994; Berardi *et al.*, 1995). Unfortunately, these markers are unavailable right now. It is also unclear where BMP-2 acts in the CNS stem cell hierarchy to increase the production of neurons from stem cell progeny. The likeliest point of action would be on a committed progenitor cell (Figure 14, solid line), though BMP-2 could also have actions on multipotent precursor cells (Figure 14, dashed line) to increase the

Figure 14:

The most plausible sites of action for BMP-2 in the CNS stem cell hierarchy.

The data presented in this thesis indicate that BMP-2 inhibits CNS stem cell proliferation and enhances the production of neurons from stem cell progeny. By assuming that the biology of CNS stem cells is controlled by a process similar to hematopoiesis and neuropoiesis, it remains unclear exactly where in the CNS stem cell hierarchy BMP-2 is acting, though some generalizations can be made. Solid arrows indicate the most probable sites of action for BMP-2, whereas dashed arrows indicate possible sites of action for BMP-2.


Totipotent Stem Cell
Inhibition of proliferation
BMP-2
Mitotically Active Precursor Cell
Differentiation
BMP-2
Committed Progenitor Cells (Mitotically Active)
Neuron

production of neurons since both populations of cells can theoretically produce neurons. Finally, BMP-2 could also act on more differentiated neurons to induce neurite outgrowth (Figure 14, dashed line), though this possibility requires more experimental evidence to make it convincing. Therefore, our results indicate that the likeliest sites of action for BMP-2 is on committed progenitor cells, to enhance the production of neurons, and both TSCs and MPCs, to inhibit their proliferation.

There are two further questions raised by this study. Are the inhibition of proliferation of CNS stem cells and the increased neuronal production from stem cell progeny somehow connected? Since there is so much uncertainty about the sites of action of BMP-2, it is difficult to speculate whether these two events are connected, though the possibility seems likely. Second, does BMP-2 act directly on the cells it appears to influence, or are its actions mediated through another cell type? This is an important issue and can only be answered by identifying the cells that are responsive to BMP-2.

Starting with the study of BDNF by Ahmed *et al.* (1995), BMP-2 is an example of another molecule with the capacity to influence the fate of CNS stem cells. Other factors that control CNS stem cells will undoubtedly be discovered. CNS stem cells may someday prove to be as valuable as hematopoietic stem cells, not only for the biologic insights they provide, but also with the therapeutic potential they represent. The goal of our lab is to reach a point where the epigenetic manipulation of CNS stem cells can be translated into the adult animal. Using extrinsic factors it may be possible to produce large numbers of neurons with a specific phenotype in the adult brain. This would undoubtedly have a large impact on the treatment of an array of neurodegenerative disorders.

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