ISOLATION, CHARACTERIZATION, AND USE OF STEM CELLS FROM THE CNS

Fred H. Gage, Jasodhara Ray, and Lisa J. Fisher Department of Neurosciences, School of Medicine, University of California, San Diego, La Jolla, California 92093-0627

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INTRODUCTION

Most of the differentiated cells in the mammalian body are not permanent. In most organs, cells are dying and being replaced at varying rates. The body has developed two strategies for tissue renewal or replacement. The first and most straightforward is duplication, wherein a differentiated cell divides to give rise to a daughter cell of the same geno- and phenotype. Hepatocytes and endothelial cells fall into this category. For hepatocytes, cell number in the adult is regulated in a predictable manner such that when cell damage occurs, the cells divide to generate the number of cells needed to replace those that have been lost. Endothelial cells are somewhat more creative in that they can divide when new blood vessels are formed. The second way that differentiated cells are replaced is by arising from undifferentiated cells through a process analogous to cell genesis. The prototypical cell of this category is the blood cell, since there is evidence that all cells of the hematopoietic lineage can be derived from a single, multipotent stem cell. These multipotent cells are capable of self-replication and the generation of various committed progenitor cells that, under the influence of appropriate cytokine and growth factors, ultimately differentiate into mature blood cells. These mature blood cells then die within a matter of weeks. Although the existence of the multipotent cell is acknowledged, it is an elusive cell whose isolation, characterization, and utilization are subjects of great interest.

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Anderson (1989) has suggested that the stem cells of the hematopoietic system are a useful analogy to apply to the nervous system and predicts that similar cells exist in both the central nervous system (CNS) and the peripheral nervous system (PNS). Although the presence and nature of multipotent cells within the CNS remain a point of discussion, the nomenclature from the hematopoeitic system has been adapted to describe neural precursors and their progeny. Stem cells thus refer to a population that is capable of extended self-renewal and the ability to generate multilineage (neurons and glia) cell types (see Figure 1). Progeny of the stem cells are progenitor cells, which are also capable of self-replication but show a limited life span. The progenitors show further lineage restriction upon division by giving rise to progeny that generate either neurons (neuroblasts) or glia (glioblasts) but not both. Although these terms are used often, there is some discrepancy in how they are defined in various studies. The problem is particularly complex in the brain because of the large number of neural phenotypes that exist. Therefore, for a cell to be called a neural stem cell, it would have to be able to differentiate not only into neurons and glia but into the vast diversity of neural cell types that are present within the CNS (e.g. interneurons, projecting neurons, type I astyrocytes). An alternative set of definitions may be less value laden and more consistent. A stem cell can be defined as a cell that (a) is not in the final stage of differentiation or terminally differentiated, (b) can continue to divide throughout the life of an animal, and (c) has progeny that can either continue as stem cells or terminally differentiate. For simplicity, since it is often difficult to determine if a cell within the CNS displays all of these features, the term precursor is used in this review to refer to any cell types that do not appear to be terminally differentiated. In some of the work summarized below, however, the terms used by different investigators are mentioned to provide a point of comparison between studies.

This review summarizes work that has sought to locate, isolate, and characterize putative stem cells within the CNS. The review is divided into several general areas. First, we discuss evidence that precursor populations normally reside within the postnatal mammalian brain and that some of these cells may continue to contribute neuronal progeny to the mature brain through adulthood. Next, we describe the characterization of neural precursor cells in vitro. Much of this work has been facilitated by the identification of factors that increase the survival and growth of primary precursor cells in vitro. Recent work that has revealed the developmental potential of cultured neural precursors when implanted back into the CNS is then discussed. We conclude by presenting work that has begun to explore the potential of neural precursors for treating CNS damage or disease. Because of the scope of this review, there are issues that will not be addressed. In particular, the controversy over the in vivo multipotentiality of progenitors and the migration patterns of neuronal progen-



- 1. How many divisions per stem cell?
- 2. What is the potentiality of stem cells?
- 3. Can stem cells persist quiescently?
- 4. How long is the cell cycle of a progenitor?
- 5. Can progenitors persist quiescently?
- What percentage of progenitors or neuroblasts die?
- 7. What is the mechanism of cell death?
- 8. How many times can a neuroblast divide?
- 9. Can a neuroblast survive indefinitely in a quiescent state?
- 10. Can neuroblasts be activated to divide in vivo?
- 11. How long do terminally differentiated cells survive?
- 12. Can a differentiated cell de-differentiate?

Figure 1 Schematic of neural stem cell lineage and questions that remain to be addressed concerning these cells and their progeny. The stem cell is capable of extended self-replication, as indicated by the arrow that loops around to the top of the stem cell. In this example, the progenitor population that arises from the stem cell is shown to generate two cells: one that dies without further commitment (circle with X) and another that is committed to the neuronal lineage (neuroblast). The neuroblast should be able to generate all of the different types of neurons within the CNS (only two types are shown). Figure adapted from figures 17–34 in Alberts et al (1989).

itors in different regions of the brain are summarized elsewhere (Hatten 1993). Rather, we have focused on the use of neural precursors to understand brain development and adult plasticity and as potential sources of cellular replacement in the damaged adult brain.

PRECURSOR POPULATIONS IN THE POSTNATAL BRAIN

The epithelium of the ectoderm gives rise to the neurons and glia that make up the adult central nervous system. The mechanisms that lead to this diversity are difficult to ascertain because cells migrate from their birth place and

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because of the large number of different cell types that make up the brain. Nevertheless, enormous headway is being made in determining how cells decide what they will become and how they find their site of terminal differentiation. Most of the mechanisms that are responsible for cell proliferation and migration are shut off in the adult mammalian brain, and thus the capacity for self repair and cellular replacement is greatly diminished. Although controversial, there is evidence that cell division continues in the adult brain and that some of the resulting cells become neurons. This controversy has been at least partially resolved over the last 30 years with the introduction of [3H]thymidine labeling techniques (Altman 1962, Sidman 1970, Rakic 1985). Quantification of those cells that incorporate [3H]thymidine into their DNA during mitosis can reveal patterns of new cell formation and the length of cell cycles. The route of administration of the label, the duration after injection to time of sacrifice, and the concentration of the isotope can all influence the interpretation of the results, but careful and methodical studies have provided a reliable set of conclusions. More recently, retroviral-mediated gene transfer has increased the arsenal of techniques that can be used to identify dividing populations within the CNS. The most common retroviral vectors contain the Escherichia coli lacZ (β -galactosidase) gene. This marker gene is integrated into the DNA of proliferating cells and then inherited by all the subsequent progeny (Sanes et al 1986, Price et al 1987). The expression of this gene is easily detected either immunocytochemically or through enzyme histochemistry with the light and electron microscope. The four areas of the adult brain that have been studied most and provide the strongest case for postnatal neurogenesis in vivo---the dentate gyrus of the hippocampus, the olfactory epithelium, the ependymal zone, and the cerebellum-are reviewed here.

Dentate Gyrus of the Hippocampus

The number of neurons in the hippocampus of the rat increases 43% between postnatal day 30 and postnatal day 365 (Bayer 1982). Two morphological changes occur in the hippocampus during this time: The overall number of cells increases, and the volume of cells in the ventral leaf of the dentate gyrus (DG) decreases. Even though pyknotic cells are rarely seen in the DG, some cell death is expected to occur. Neural precursors appear to arise from a stem cell population that lies in the basal region of the granule cell layer. This has been suggested from labeling studies that reveal marked cells in this region shortly after [³H]thymidine pulsing and few cells in the granule cell layer when the area is examined at longer intervals after labeling. Convincing evidence from several studies indicates that this dividing cell population develops into mature neurons. Using electron microscopy and [³H]thymidine autoradiography, Kaplan (1981) observed that in the postnatal dentate gyrus there are mitotic neuroblasts in prophase and telophase with synapses on their cell bodies

Antigenic markers	Cell types	Nature of antigens	Reference		
Nestin	Stem	Intermediate filament	Fredriksen & McKay 1988		
Vimentin	Precursor	Intermediate filament	Houle & Fedoroff 1983		
A2B5	Precursor	Ganglioside	Eisenbarth et al 1979		
LI	Premigratory neuron	Cell adhesion molecule	Rathjen & Schachner 1984		
NF (L,M,H)	Neuron	Intermediate filament	Carden et al 1987		
NSE	Neuron	Enolase (isoenzyme)	Schmechel et al 1980		
MAP-2	Neuron	Microtubule-associated protein	Dotti et al 1987		
Calbindin-D	Neuron	Ca ²⁺ binding protein	Sloviter 1989		
Substance P	Neuron	Peptide	Cooper et al 1982		
GABA	Neuron and glia	Amino acid	Cooper et al 1982		
GFAP	Astrocyte	Intermediate filament	Debus et al 1983		
Gal C	Oligodendrocyte	Galactosphingolipid	Ranscht et al 1982		
O4	Oligodendrocyte	Sulfatide	Sommer & Schachner 1981		

Table 1 Immunocytochemical markers used to identify neuronal and glial cells

and with axonal-like processes. Stanfield & Trice (1988) labeled cells with ³H)thymidine, allowed them to mature for longer than a month, and then injected fluorescent dye into CA3, the terminal field of dentate granule neurons. They observed that some of the thymidine-labeled cells also contained the fluorescent dye, indicating that the newborn cells displayed the characteristic neuronal feature of an axonal process. More recently, Cameron et al (1993) examined the timecourse of the maturation of the proliferative population within the hippocampus by pulsing the adult brain with [3H]thymidine and then using combined autoradiography and immunolabeling for neuron-specific enolase (NSE) and the glial marker glial fibrillary acidic protein (GFAP) (see Table 1). Their results showed that the number of cells double-labeled for NSE and [³H]thymidine progressively increased after the thymidine injection; 85% of the labeled cells were NSE positive four weeks postlabeling. In contrast, the number of dual-labeled GFAP cells remained constant across the same four-week time period. The authors concluded that the majority of newborn cells in the dentate gyrus differentiate into neurons, not glia, and that some of the cells are born in the granule cell layer while others migrate from the hilus to the granule cell layer.

The significance of neurogenesis in the adult hippocampus is presently unknown. It is perhaps too easy to speculate that this neuronal plasticity may be important in a structure that is involved in the formation of new memories. Perhaps, unlike areas of the brain that are involved in long-term memory, there may be an advantage to having cell replacement and addition occur in areas involved in cognitive plasticity. Clearly, the functional ramifications of neurogenesis within the hippocampus remain to be elucidated.

Olfactory Epithelium

In the olfactory epithelium, the sensory neurons undergo continual degeneration and replacement into adulthood (Graziadei & Monti Graziadei 1978, 1979). The neurons are generated from two basal unipotent stem cell populations-the horizontal basal cell and the globose basal cell, which exists in the base of the epithelium (Kratzing 1978, Graziadei et al 1980). In most mammals, the lifetime of an olfactory cell lasts about one month; this includes the birth, migration, synaptogenesis, and degeneration of these neurons. The factors that regulate this cell cycle are unknown, but it has been hypothesized that the rate of cell birth is regulated by death of the mature neurons. Furthermore, the final differentiation and survival of olfactory neurons is predicted to be dependent on synaptic contact with target cells in the olfactory bulb. Studies showing that damage to mature cells of the epithelium by a variety of manipulations can result in an increase in olfactory neuronal production have provided evidence that supports the role of cell death in olfactory neurogenesis (Matulonius 1975, Graziadei et al 1979, Camara & Harding 1984, Verhaagen et al 1990). Schwartz et al (1991) showed that only the globose basal cells contribute to the neurogenesis that occurs following experimental damage (bulbectomy). Although bulbectomy can increase the rate of cell neurogenesis in the olfactory epithelium, a parallel increase in the rate of cell death also occurs (Carr & Farbman 1992). This latter observation is consistent with a role of target cells in maintaining the survival of differentiated neurons within the olfactory bulb.

Subependymal Zone

In the embryo, proliferation occurs in the ventricular zone, a cellular lining of the ventricles that generates postmitotic cells that migrate from the ventricular zone to form the forebrain. The subventricular zone expands greatly during late gestation and early postnatal periods and is a germinal matrix of the immature dividing neuroectodermal cells. The subventricular zone persists in the adult as a mitotically active area called the subependymal zone (Boulder Committee 1970).

The fate of the subependymal cells is not fully understood. Many recent retroviral lineage-tracer studies are not conclusive, but several intriguing issues important to the isolation of stem cells have been revealed. Levison & Goldman (1993) gave postnatal day 2 (P2) rats injections of a retrovirus harboring either β -galactosidase (β -gal) or alkaline phosphatase, or both. Their results demonstrate that both astroctyes and oligodendrocytes are born in the subependymal zone postnatally. Furthermore, when two different markers were used, evidence supported the conclusion that a single progenitor from the subependymal zone produces both types of glia. Other work by Luskin (1993) presents evidence that the postnatal subependymal zone is heterogeneous. In this study, a retroviral tracer was injected into different parts of the subependymal zone of postnatal rats to reveal the presence of marked cells and cell clusters at various times after the injection. She found that a discrete region of the anterior part of the subependymal zone that lies at the anterior pole generated a large number of neurons that differentiated into granule cells and periglomerular cells of the olfactory bulb. These results are in agreement with a recent report that neurogenesis of olfactory bulb interneurons occurs postnatally, and for granule cells, it extends for many months (Corotto et al 1993).

The ability of the subventricular zone to generate glia and neurons has been suggested to decrease with age. Morshead & van der Kooy (1992) used a retroviral tracer to label mitotically active cells in the adult mouse brain and then looked for migration of labeled progeny out of the subependymal zone and into the surrounding brain tissue. They concluded that the proliferating cells of the subependymal zone self-replicate and also generate progeny that die soon after birth without apparent migration from their birth site. However, Lois & Alvarez-Buylla (1994) recently provided convincing evidence that the subventricular zone is capable of generating neuronal progeny for extended periods in the adult brain. Their discrete microinjections of $[^{3}H]$ thymidine into the lateral ventricles of adult mice revealed clusters of proliferative cells in the subventricular zone that migrated to the olfactory bulb over distances as great as 5 mm before differentiating into either granule neurons or periglomerular cells. Thus even in the mature brain, the subventricular zone harbors an active precursor population that contributes some of the neuronal cell types that reside within the olfactory bulb.

Cerebellum

The five major neuronal cell types of the cerebellum have a distinct spatial and temporal developmental pattern. There are two separate waves of development originating from two separate germinal zones. The deep cerebellar neurons, the Purkinje cells, the Golgi II, and the Golgi epithelial cells all emerge from a zone in the roof of the fourth ventricle called the rhombic lip. A second wave of cell birth occurs later in development and extends into the postnatal period in most vertebrate species. This second germinal zone, termed the external germinal layer (EGL), is formed immediately beneath the pia covering the cerebellar plate and gives rise to granule cells, stellate cells, basket cells, and some glial cells (Addison 1911, Raaf & Kernohan 1944, Phemister & Young 1968). The ease of localizing, dissecting, and manipulating the EGL has made it a focus of developmental studies. In all vertebrates, the EGL expands from a single layer to six to eight cell layers during cell mitogenesis. The EGL persists for varying periods in different species and eventually reduces in size and disappears. For example, the EGL is seen up through P25 in rodents (Addison 1911), to approximately P70 in the dog (Phemister &

Young 1968), and for almost two years in the human (Raaf & Kernohan 1944). Although the proliferative cells in the EGL do not appear to survive as a self-replicating population throughout the life of an organism, potential stem cells derived from the EGL may become quiescent in the maturing CNS but retain the capacity for proliferation if exposed to appropriate environmental signals.

Other

Other areas of the adult brain have been demonstrated to undergo cell division; however, less information and fewer published reports are available about them. Kaplan (1981) reported that 30 days after a three-month-old rat was injected with [³H]thymidine, labeled cells were observed in layer IV of the visual cortex. Ultrastructurally, these labeled cells had the morphology of neurons, including synapses along their dendrites and axons. Although this labeling accounted for only .011% of the cells per section through the entire thickness of the visual cortex, it represents an important population whose neurogenesis in the adult could be significant. There are probably many areas of the adult brain undergoing neurogenesis at a slow rate or low frequency that, when examined in detail, could represent the presence of quiescent stem cells activated for reasons that are presently unknown.

IN VITRO CHARACTERIZATIONS OF PRECURSOR CELLS

One of the first studies that appeared to isolate a multipotent cell in vitro was conducted by Price et al (1987), who explored the characteristics of cells cultured from the embryonic cortex of rats that were transduced to express the β -gal gene. Using morphological criteria, Price et al (1987) demonstrated that discrete clusters of labeled cells that presumably arose from a single β -gal-expressing cell appeared to contain multiple cell types. This observation was subsequently confirmed and extended by Williams and colleagues (1991), who have reported that 18% of the cells within the embryonic cortex generate both neurons and oligodendrocytes in vitro.

An early characterization of multipotent cells isolated from the CNS revealed an influence of exogenous factors on these precursor populations (Temple 1989). Cells derived from the embryonic septum of rats were placed into culture either alone or in combination with fetal striatal tissue. Nearly 50% of the septal cells in the cocultures were found to proliferate for extended periods, whereas none of those cultured alone was capable of more than one division. Some isolated clones of proliferating cells were clearly multipotent, as indicated by the emergence of mixed progeny that expressed either the glial marker GFAP or the neuronal marker neurofilament (NF). This finding indicated that

a soluble factor produced within the developing CNS strongly influences the mitogenesis of neural precursors. Similar results obtained with single cells isolated from the embryonic cortex and hippocampus suggested that this response was not unique to precursors derived from a particular region of the CNS. In the following section, studies that have identified multipotent precursor cells and sought to identify some of the exogenous factors that influence these precursor populations in vitro are discussed. Although the focus of this review is on CNS-derived cells, we have included some work on peripheral precursors that has contributed to an understanding of trophic and substrate influences on stem cells.

Immortalized Neural Populations

Historically, it has been difficult to maintain the survival and/or proliferation of primary neural precursors in vitro for periods sufficiently long enough to characterize them and examine cellular responses to exogenous factors. Therefore, many groups have developed neural cell lines by using gene transfer techniques to model precursor responsiveness to experimental manipulations. In addition to being able to readily expand these cell lines for study, the cells can be cloned to obtain homogeneous populations of precursors and enhance reproducibility.

Neural cell lines have typically been generated by the retroviral transduction of oncogenes into cells derived from the developing brain (for review see Cepko 1988, 1989; Lendhal & McKay 1990). The immortalization process arrests cells at defined stages of development and generally halts terminal differentiation. Thus, cells at intermediate stages of differentiation can be propagated for a long period of time. The most commonly used oncogenes for immortalizing cells are members of the *myc* oncogene family (Cepko 1988, 1989) or a temperature-sensitive (ts) mutant of SV40 large T antigen (Jat & Sharp 1989). In contrast to the *myc* oncogene, the large T antigen gene induces proliferation when cells are maintained at 33°C (permissive temperature) but not at 39°C (nonpermissive temperature). Interestingly, not all cells expressing an oncogene become immortal (Ryder et al 1990). It is unclear why some oncogene-expressing cells display a limited life span, but this may reflect an inability of the oncogene to override a differentiation program that was initiated prior to the immortalization.

Several groups have reported the presence of multipotent cells in cultures of immortalized neural populations. Ryder and colleagues (1990) described *myc*-immortalized clones derived from the cerebral cortex or olfactory bulbs of early postnatal rats that generate neuronal and glial progeny. Similarly, immortalized populations derived from the mesencephalon of embyronic day 10 (E10) mice generate multiple cell types in vitro (Bernard et al 1989). That the diverse progeny arose from a single precursor was confirmed by showing

a unique viral integration site for each clonal line (Bernard et al 1989, Ryder et al 1990). These multipotent populations did not appear to be an abnormal consequence of the immortalization procedure because many other myc clones failed to spontaneously generate multiple cell types (Bartlett et al 1988, Cepko 1989, Ryder et al 1990). At least some of the myc-immortalized clones have been found to be responsive to exogenous stimuli. One of the precursor lines that remained in an undifferentiated state in vitro could be induced to differentiate within 24 hours of the addition of acidic fibroblast growth factor (aFGF) or basic FGF (bFGF) to the culture medium (Bartlett et al 1988). Some of the progeny that arose in the FGF-enriched environment displayed NF protein, whereas others expressed GFAP, indicating the generation of both neurons and glia from the precursor population. In some of the lines that showed spontaneous multipotency in vitro, members of the FGF family could induce a rapid increase in NF expression (Bernard et al 1989). In addition, substances secreted from some of the myc-clones that did not appear to be in the FGF family have been found to be mitogenic for immortalized precursors (Bernard et al 1989). All of these results must be interpreted with caution, however, since diverse culture conditions are used in different studies (see Table 2).

In contrast to the myc-expressing clones, cells immortalized with the tsSV40 large T antigen often do not show spontaneous multipotency in vitro. T antigen-expressing cells derived from the cerebellum, striatum, or medullary raphe of rodents typically generate progeny that express either glial markers (Fredriksen et al 1988, Evard et al 1990, Redies et al 1991) or neuronal phenotypes (Redies et al 1991, White & Whittemore 1992) but not both. In many of these clonal lines, alterations in the morphology and/or phenotype of T antigen-expressing clones can be induced by manipulating the extracellular environment of the cells in vitro. The cerebellar-derived clonal line ST15A, characterized by a neuronal morphology and expression of NF, shows conversion to a GFAP phenotype when the cells are cocultured with primary cells derived from the P3 cerebellum (Redies et al 1991). A cell line derived from the P1 striatum of mice that solely showed gliogenesis in a serum-enriched medium generated both NF-expressing cells (neurons) and glia when switched to a chemically defined medium and a polyornithine substrate (Evard et al 1990). Also, both the survival and differentiation of a clonal medullary raphe population (RN33B) are enhanced when the cells are cocultured with embryonic hippocampus or cortex tissues (Whittemore & White 1993). Furthermore, changes in substratum affect the differentiation of RN33B and the expression of neuronal antigens (Whittemore & White 1993). Cells grown on poly-D-lysine show reduced levels of neuron-specific enolase (NSE) and a complete abolition of NF immunoreactivity. In contrast, laminin and fibronectin (FN) both slightly enhance the NSE expression of RN33B cells without affecting NF. The influence of exogenous factors on the T antigen-expressing cells is

Species	Region/Age	Cell type ^a	Immortalizing oncogene	Substratum	Medium	Supplement	Exogenous factor	Reference
Mouse	Cerebellum/P4	Multipotent progenitor	v-myc	Poly-L-lysine	DME ^b + 20% FBS	No	No	Ryder et al 1990
Mouse	Olfactory bulb/ newborn	Multipotent progenitor	v-myc	Poly-L-lysine	DME + 20% FBS	No	No	Ryder et al 1990
Mouse	Mesencephalon/E10	Precursor	c-myc and N-myc	Uncoated plastic	DME + 10% FBS	No	No	Bartlett et al 1988, Bernard et al 1989
Mouse	Striatum/newborn	Bipotential precursor	SV 40 large T antigen	Uncoated plastic	DME + 10% FBS	No	No	Evard et al 1990
Rat	Adrenal gland/E14.5	SA progenitor MAH	v-myc	Uncoated plastic	L-15 + 10% FBS	Dexamethasone + additives ^c	No	Birren & Anderson 1990
Rat	Cerebellum/P2	Oligopotent precursor	tsSV40 A58	PORNd	DME + 10% FBS	No	No	Fredriksen et al 1988
Rat	Cerebellum/P2	Oligopotent precursor	tsSV40 A58	PORN and Laminin	DME + 10% FBS	No	No	Redies et al 1991
Rat	Hippocampus/E16	Stem	tsSV40 A58	PORN	DME + 10% FBS	No	No	Refranz et al 1991
Rat	Medullary raphe/E13	Neuroblast RN33B	tsSV40 A58	Collagen and Poly-L- lysine	DME:F-12 + 10% FBS	No	No	Whittemore & White 1993
Rat	Neural tube/E10.5	Neural crest progenitor NCM-1	v-myc	Fibronectin	L-15 + 10% FBS	No	No	Lo et al 1991

Table 2 Culture conditions for immortalized stem and precursor cells

^aCell types as defined by authors.

^bDME = Dulbecco's modified eagles medium.

^c Doupe et al 1985.

^dPORN = Polyomithine.

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consistent with results obtained with myc clones, thus supporting the use of immortalized precursors as a model system to explore CNS development. In general, these studies have revealed a role for both cell surface cues and soluble molecules in the proliferation and differentiation of neural precursors.

Immortalized Cells Derived from the PNS

Sympathoadrenal (SA) precursor cells of the peripheral nervous system are bipotential and differentiate into adrenal chromaffin cells or sympathetic neurons depending on environmental influences. To elucidate the exogenous factors that influence the fate choice and the differentiation of SA progenitor cells in vitro, a v-myc immortalized cell line, termed MAH (myc immortalized, adrenal derived, HNK-1 positive), was generated from the E14.5 adrenal gland (Birren & Anderson 1990). This cell line displays many of the properties of the SA precursors in situ and has thus been used to explore the development of nerve growth factor (NGF) responsiveness in the sympathoadrenal lineage. In vitro, NGF induces chromaffin cells to show the morphological and antigenic characteristics typical of sympathetic neurons (Doupe et al 1985). However, the SA progenitors are unresponsive to NGF (Anderson & Axel 1986). Like their in situ counterparts, MAH cells do not respond to NGF in vitro, a property that appears to reflect the absence of both the low- (p75) and highaffinity (trkA) forms of the NGF receptor in this SA cell line (Birren & Anderson 1990, Birren et al 1992). Birren & Anderson (1990) have found that bFGF is mitogenic for MAH cells and induces neurite outgrowth and expression of p75. Once the NGF receptor is expressed on these cells, they then become dependent on NGF for continued survival. These results indicate that NGF is not the initial determinant of neuronal differentiation in the SA lineage, but rather bFGF plays this role (see also Stemple et al 1988).

Lo et al (1991) have suggested that another clonal cell line generated by immortalization of neural crest cells with *v-myc*, termed NCM-1 cells, is a glial progenitor population. These cells spontaneously differentiate into Schwann cells in vitro, a process that can be blocked by culturing the progenitors in the mitogenic factor TGF- β (transforming growth factor- β). Some subclones of NCM-1 cells generate progeny that do not express glial markers. Rather, the subclones give rise to cells that show features characteristic of SA progenitors, such as the induction of tyrosine hydroxylase and NF in response to bFGF and dexamethasone. Thus, NCM-1 cells may represent multipotent neural crest stem cells that can generate both glial precursors and SA progenitor-like cells depending on environmental signals.

Primary Precursor Cells

Although the cellular immortalization technique offers many advantages, this process may alter some of the fundamental properties of the parent cells.

Several studies have reported that differences in these two cell types exist. Immortalized cells have shown altered expression of some proteins that are characteristic of the original parent population (Birren & Anderson 1990, Renfranz et al 1991, Vandenberg et al 1991, Whittemore & White 1993). Also, the growth rate of immortalized cells is often faster than that of their primary counterpart (Ryder et al 1990), a property that can be reflected in abnormal karyotypes (Bianchi et al 1993). Thus, many recent studies have focused on characterizing the properties of primary precursor cells in vitro. Much of this work has been aided by the identification of growth factors that enhance the survival and/or proliferation of primary precursors in culture. For comparative purposes, the divergent culture conditions used to examine primary cells in the studies discussed below are listed in Table 3.

Primary Precursor Cells in the Developing Neural Crest

Stemple & Anderson (1992) examined the developmental potentials of serially propagated mammalian neural crest cells in vitro. They found that single neural crest cells are multipotent and able to produce multipotent progeny, indicating that these cells are capable of self renewal and are thus likely to be stem cells. Assessments of the cells in different plating conditions have revealed that substratum plays a role in the fate choice of these stem cells. This was first suggested by observations that neural crest clones that were first established on FN and then overlaid with poly-D-lysine (pDL) at various times postplating gave rise to neuron-only clones more often than glia-only clones (Stemple & Anderson 1992). To demonstrate that the substratum was influencing the lineage decision of the stem cells, clones established on FN were picked and replated at clonal density on either FN or pDL-FN substrates. Subclones of the FN-derived founder were seen to generate neurons on the pDL-FN substratum, but sister cultures plated onto FN generated only glial cells. Although exposure to pDL appeared to drive the precursor population toward a neuronal lineage, this was not always the case. For example, clones founded on pDL-FN and then subcultured were only capable of generating neurons when the FN substratum was enriched with pDL. These results suggest that neural crest cells exposed to FN retain the capacity for neurogenesis and that pDL influences, but does not dictate, neuronal differentiation of these stem cells. Growth factors can also influence or bias the lineage choice of uncommitted neural crest stem cells. Glial growth factor (GGF), also known as Schwann cell-mitogen, suppresses neuronal differentiation while allowing or promoting glial differentiation (Shah et al 1994).

Glial Precursor Cells in the Optic Nerve

The rat optic nerve contains three different types of glial cells: type 1 astrocytes, type 2 astrocytes, and oligodendrocytes. Two of these populations, the

Species	Region/Age	Cell type ^a	Substratum	Medium	Supplement	Exogenous factors	References
Mouse	Mesencephalon and Telencephalon/E10	Precursor	Uncoated plastic	DME + 1% FBS	N2 ⁶	bFGF (50 ng/ml) and heparin (8 μg/ml)	Murphy et al 1990, Drago et al 1991a,b
Mouse	Mesencephalon and Telencephalon/E10	Precursor	Uncoated plastic	Monomed + 10% FBS	No	bFGF (20 ng/ml) and heparin (8 μg/ml)	Kilpatrick & Bartlett 1993
Mouse	Striatum/EI 4	Multipotent progenitor	PORN	DME: F-12	N2	EGF (20 ng/ml)	Reynolds et al 1992, Vescovi et al 1993
Rat	Cerebral hemispheres or spinal cord E13-14	Neuroblast	Poly-L-lysine	DME	N2	bFGF (5 ng/ml)	Gensburger et al 1987, DelouIme et al 1991
Rat	Cortex/E15	Multipotent precursor	Astrocyte monolayer	DME + 10% FBS	No	No	Price et al 1987
Rat	Cortex/E12-18	Multipotent precursor	Astrocyte monolayer	DME + 0.5% FBS	N2	No	Williams et al 1991
Rat	Hippocampus or spinal cord/E16-18	Neuroblast	PORN and Laminin	DME : F-12	N2	bFGF (20 ng/ml)	Ray et al 1993, Ray & Gage 1994
Rat	Neural crest/E10.5	Stem	Fibronectin	L-15	N2 + additives ^c	EGF (100 ng/ml), bFGF (4 ng/ml), and NGF (20 ng/ml)	Stemple & Anderson 1992
Rat	Optic nerve/PO-adult	O-2A progenitor	Poly-L-lysine	DME + 0.5% FBS	N2	No	Wilswijk et al 1990, Wren et al 1992
Rat	Striatum/E13.5-14.5	Stem	PORN	DME: F-12	N2	bFGF (5 ng/ml), NGF (150 ng/ml), or bFGF \rightarrow NGF	Cattaneo & McKay 1990
Mouse	Brain/Adult	Precursor	Uncoated plastic	DME + 10% FBS	No	bFGF (20 ng/ml), EGF (20 ng/ml), or Ast-1 CM ^d	Richards et al 1992
Mouse	Striatum/Adult	Stem	Uncoated plastic	DME : F-12	N2	EGF (20 ng/ml)	Reynolds & Weiss 1992
Rat	Hippocampus/Adult	Neuroblast	Uncoated plastic	DME: F-12	N2	bFGF (20 ng/nl)	Gage et al 1994

Table 3 Culture conditions for primary stem and precursor cells

^a Cell types as defined by authors.

^b N2 = Bottenstein & Sato 1980.

Sieber-Blum & Chokshi 1985.

 d CM = Conditioned medium.

type 2 astrocytes and oligodendrocytes, have a common precursor that has been named the O-2A progenitor (Raff et al 1983). Both the proliferation and differentiation of O-2A progenitors isolated from the neonatal optic nerve are influenced by exogenous factors. In an early in vitro study, the progenitor population showed strong mitogenic activity when cocultured with type 1 astrocytes (Noble & Murray 1984). It was subsequently determined that these astrocytes secrete platelet-derived growth factor (PDGF), which induces the proliferation of O-2A cells in culture (Noble et al 1988). However, even in the presence of PDGF, the progenitor cells eventually differentiate spontaneously and give rise to the two glial cell types. This terminal differentiation can be blocked by further exposing the O-2A cells to bFGF, which traps them into a self-renewal loop for prolonged periods (Bögler et al 1990). The type of glial cell generated from the progenitor varies depending on the presence of serum in the culture media; type 2 astrocytes arise in serum-rich environments, whereas oligodendrocytes are generated in serum-poor environments. Another factor that can promote O-2A differentiation into type 2 astrocytes is ciliary neurotrophic factor (Hughes et al 1988). These in vitro studies thus support a role for endogenous factors in glial cell development.

Interestingly, bipotent O-2A progenitors are evident within the optic nerve into adulthood (Wolswijk & Noble 1989). However, these adult progenitors display a different morphology, cell cycle length, and migration rate than O-2A cells found within the perinatal optic nerve. A number of observations suggest that the neonatal O-2A cells in the adult optic nerve are completely replaced with a self-renewing, adult form of the progenitor. For example, perinatal O-2A cells serially passaged for 3 months gradually convert to cells that express a marker (O4 antigen) characteristic of adult precursors (Wren et al 1992), and the number of O4-positive cells within the optic nerve in vivo increases from <2% at P7 to >95% in 8-month-old or older rats (Wolswijk et al 1990). These observations further confirm the presence of precursor cells within the adult CNS and also indicate that precursors can show altered properties as the nervous system develops.

Effects of Epidermal Growth Factor on Embryonic Neural Precursors

The mitogenic growth factor epidermal growth factor (EGF) has proliferative effects on neural cells isolated from the embryonic (E14) mouse striatum (Reynolds et al 1992). These EGF-responsive cells, which have been suggested to be a progenitor population within the CNS, also proliferate in the presence of TGF- α but are unresponsive to NGF, bFGF, PDGF, or TGF- β . EGF can induce the proliferation of a single progenitor cell plated on polyornithine (PORN) substratum in serum-free culture conditions and can generate a cluster of undifferentiated progeny that solely express nestin (Reynolds et al 1992),

a marker of intermediate filaments within neuroepithelial cells during development (Fredricksen & McKay 1988, Lendahl et al 1990). After multiple cell divisions, cells migrate from the clusters and give rise to NSE-immunoreactive (IR) or GFAP-IR cells. In addition, new proliferating clusters of cells are also seen. The secondary clusters that form in the EGF-enriched culture contain cells with the same undifferentiated morphology as the original progenitor, suggesting the presence of a self-renewing population of cells. Many of the NSE-IR cells also express NF protein, and a small number of cells with neuronal morphologies express markers characteristic of striatal neurons, such as GABA, substance P, and methionine-enkephalin. Since multiple types of progeny are generated in EGF-enriched conditions, these results support a role for EGF in mitosis but not in differentiation of the precursors (see below).

Effects of bFGF on Embryonic Neural Precursors

Catteneo & McKay (1990) isolated cells from E13.5–14.5 rat striatum that expressed nestin, which were termed a stem cell population. In serum-free conditions, bFGF promoted both the survival and proliferation of these precursors in vitro, an effect that was potentiated by NGF. Removal of growth factors halted the proliferation of the precursors, which then differentiated into cells that displayed neuronal morphologies and expression of NF. The proliferating precursors may thus have represented a neuroblast population, rather than stem cells, because they appeared to give rise solely to neurons. The mitogenic action of bFGF on neuroblasts is not restricted to cells isolated from the striatum; similar results have also been observed in cultures derived from embryonic cerebral hemispheres (E13), hippocampus (E16), and spinal cord (E14-16) of rats (Gensburger et al 1987, Deloulme et al 1991, Ray et al 1993, Ray & Gage 1994). Cells in all of these regions that proliferate in response to bFGF generate progeny that predominantly express neuronal markers such as NF, NSE, and microtubule-associated protein-2 (MAP-2) (Gensburger et al 1987, Deloulme et al 1991, Ray et al 1993, Ray & Gage 1994).

The mitogenic action of bFGF is not restricted to neuroblasts; multipotent precursors isolated from the telencephalon and mesencephalon of embryonic (E10) mice also proliferate in response to the growth factor (Kilpatrick & Bartlett 1993). In serum-enriched conditions supplemented with bFGF, two types of clones are generated by these precursors: type A cells (37% of the clones) with a large, amorphous morphology, and type B cells (54% of the clones) that display a cubodial, epithelial morphology. Among the proliferative type B clones, approximately 12% generated only neurons, whereas 39% generated a mixture of undifferentiated cells, neurons, and astrocytes. The undifferentiated progeny of this latter population were also capable of generating neuronal and glial cell types, which suggests that the proliferating parent cell may be a neural stem cell. Unlike the striatal cells characterized by

Cattaneo & McKay (1990), the differentiation of multipotent precursors did not appear to be influenced by the removal of bFGF (Murphy et al 1990, Kilpatrick & Bartlett 1993). These disparate results may reflect phylogenic or ontogenic differences or differing culture conditions between the studies. The study of rat striatal cells used defined serum-free medium (Catteneo & McKay 1990), whereas the studies with neuroepithelial cells used 10% FBS. Alternatively, these differences may reflect the properties of two different precursor populations: stem cells (Kilpatrick & Bartlett 1993) and neuroblasts (Catteneo & McKay 1990). The multipotent bFGF-responsive cells were also completely unaffected by EGF (Kilpatrick & Bartlett 1993). This contrasts with the results of Reynolds and colleagues (Reynolds et al 1992) and may reflect a difference in age between the two putative neural stem cell populations or the fact that multipotent cells in different brain regions respond differently to epigenetic signals.

Evidence that a cascade of growth factors may differentially influence the proliferation and differentiation of multipotent cells has been suggested from work with the EGF-responsive striatal precursors. Although bFGF was not mitogenic for this population (Reynolds et al 1992), it did appear to strongly influence the differentiation of the cells (Vescovi et al 1993). Transient exposure of EGF-generated cell clusters to bFGF in a serum-enriched environment generated two different types of secondary progenitor cells. One was a bipotential cell (5% of the total population) that gave rise to cells with morphological characteristics and antigenic properties of neurons and astrocytes. The other cell type (4% of the total cell population) appeared to be neuroblasts that generated only neurons. Thus, bFGF appeared to be involved in the generation of secondary progenitor populations from the original precursors that were initially unresponsive to bFGF (Reynolds et al 1992).

Interestingly, the presence of serum is essential for achieving both the proliferative and the differentiation effects of bFGF on multipotent precursors (Kilpatrick & Bartlett 1993, Vescovi et al 1993). This finding indicates that there are other factors that supplement and/or potentiate the effects of bFGF on neural precursors. Indeed, bFGF-induced proliferation of neuroepithelial cells has been reported to be dependent on the presence of insulin-like growth factor I (IGF I) (Drago et al 1991a). IGF I appears to act primarily as a survival factor that increases the number of healthy cells that can respond to the mitogenic action of bFGF. The synthesis of laminin, an extracellular matrix protein, is also upregulated by bFGF in a subpopulation of glial precursors (Drago et al 1991b). Laminin appears to act in a paracrine manner to stimulate the differentiation of neuroepithelial cells. Drago et al (1991b) have demonstrated this by showing that antibodies to laminin block the effects of exogenously added laminin on neuroepithelial cell proliferation and neuronal differentiation. These results indicate that bFGF is mitogenic for neural pre-

cursors but that the survival and differentiation of cells in bFGF-enriched environments are secondary and are most likely regulated by cellular factors produced in response to bFGF.

Primary Precursor Cells from the Adult Brain

The isolation of precursor cells from the adult brain has been reported by a number of groups (Reynolds & Weiss 1992, Richards et al 1992, Lois & Alvarez-Buylla 1993). This finding is not completely unexpected, since neurogenesis does continue through adulthood in some parts of the brain (see above). Perhaps more striking are observations that these adult precursors respond to many of the same exogenous substances that influence multipotent precursors isolated from the developing CNS. Such results support the possibility that a resident stem cell population within the CNS persists through the lifetime of an organism.

Effects of EGF on Adult Neural Precursors

Reynolds & Weiss (1992) have identified a multipotent population of cells derived from the striatum of adult mice (3-18 months old) in EGF-enriched cultures. These cells proliferate in response to EGF and are unresponsive to bFGF, NGF, or PDGF. The EGF-responsive cells form spheres of proliferating cells after 6–8 days in vitro (DIV) on uncoated substratum. These cells express nestin but no neural markers. Cells within these primary spheres can continue to proliferate in secondary cultures when the primary spheres are dissociated and replated as single cells. Cells that migrate out from the spheres can develop morphologically and express antigenic markers for neurons and astrocytes. The similarities between the EGF-responsive precursors isolated from the adult striatum and the population derived from the fetal striatum (Reynolds et al 1992) suggest that these cells may represent a persistent population of neural stem cells. Although the precise location of these cells within the brain is unknown, they may arise from the subependymal zone, a region that has been reported to harbor stem cells in the adult mouse brain (Lois & Alvarez-Buylla 1993; see above).

Effects of bFGF on Adult Neural Precursors

Another growth factor that stimulates the proliferation of precursor cells from the adult (>60 days) mouse brain in vitro is bFGF (Richards et al 1992). Cells have been cultured in serum-enriched or serum-free conditions supplemented with bFGF, EGF, or both. In some experiments, conditioned medium collected from the astrocyte cell line Ast-1 has also been used in conjunction with the growth factors. In all of the culture conditions, astocytes were the most prevalent cell type that appeared to be generated. Neurons were most frequently observed in cultures containing Ast-1 medium and bFGF. The origin of these growth factor-responsive cells remains to be clarified.

Cells isolated from the hippocampus of adult (>3 months) rats also show a proliferative response to bFGF (Gage et al 1994). Using bromodeoxyuridine (BrdU) incorporation as an index of cellular proliferation, both neurons and astrocytes are generated in these cultures for extended periods (>200 DIV). However, there is an increasing proportion of neuronal-like cells in long-term cultures, suggesting that bFGF is predominantly stimulating the proliferation of neuroblasts. Such a possibility is consistent with the effect of bFGF on neural precursors isolated from the embryonic brain (see above). Alternatively, bFGF may act as a differentiation factor that drives undifferentiated precursors toward a neuronal lineage, as has been observed in the sympathoadrenal system (see above).

Taken together, the in vitro studies of neural precursors from the embryonic and adult CNS have indicated that although genetic factors may play a role in the fate choice and development of a cell, environmental factors are also important components in these processes. A number of exogenous signals, such as growth factors, have clearly been implicated in the development and differentiation of progeny arising from precursor cells. In addition, recent studies examining the effects of growth factors on cells cultured from the adult brain have demonstrated that a population of embryonic stem cells survives through adulthood and that factors that influence precursors early in development appear to remain important into adulthood.

IN VIVO CHARACTERIZATIONS OF NEURAL PRECURSORS

Characterizations of neural precursors in vitro have identified growth factors and substrates that influence cellular proliferation and differentiation. More recently, precursor populations have been implanted back into the brain to assess the developmental plasticity of these cells in different environmental conditions in vivo. Although there is an extensive literature that has explored the properties of heterogeneous mixtures of fetal neural tissues implanted into various regions of the CNS (see Fisher & Gage 1993), this portion of the review focuses on studies that have assessed the developmental potential of isolated and well-characterized populations of neural precursors.

Immortalized Hippocampal Precursors

Cells obtained from the embryonic rat hippocampus during active neurogenesis (E16) have been immortalized using the temperature-sensitive oncogene described above (Renfranz et al 1991). A clonal population selected from this immortalized population, named HiB5 cells, shows some evidence of differ-

entiation at 39°C by the formation of multiple processes but fails to express proteins that are characteristic of either glial (GFAP) or neuronal (NF) populations. However, some similarities between the immortal cells and the primary hippocampal precursors in vitro suggested that the HiB5 cells would provide a model population for exploring the growth and plasticity of neural precursors in the developing CNS. It was of particular interest to assess the fate decisions of the immortalized hippocampal cells when exposed to regions of the postnatal brain that show active neurogenesis. The HiB5 cells were therefore labeled with [3H]thymidine or fluorescent markers, implanted into the hippocampus or cerebellum of rats on postnatal day 2, and assessed 1-6 weeks postgrafting. Regardless of the transplantation site, the vast majority of labeled HiB5 cells were associated with the proliferative subregions of these areas. Specifically, HiB5 cells were predominantly incorporated into the granule cell layer of the dentate gyrus or into the granular layer of the cerebellum. Labeled HiB5 cells in the dentate gyrus displayed ovoid somata, a rich dendritic outgrowth into the molecular layer and an axon that was often found to extend through the mossy fiber pathway to the CA3 region. These engrafted HiB5 cells also expressed the marker calbindin and upregulated *c-fos* in response to kainic acid injections (McKay 1992). These combined properties are characteristic of endogenous granule neurons within the dentate gyrus (Feldmann & Christakos 1983, Smeyne et al 1992), suggesting that the immortalized precursors were driven by cues in the extracellular environment to differentiate into the newly emerging granule cell phenotype.

The HiB5 cells implanted into the cerebellum displayed markedly different properties from those transplanted into the hippocampus (Renfranz et al 1991). The immortalized cells placed into the cerebellum showed over a twofold increase in cell number after grafting, whereas less than 25% of the HiB5 cells implanted into the hippocampus survived. These findings may suggest that there are factors within the postnatal cerebellum that promote the proliferation of neural precursors. Morphological assessments of cells engrafted within the cerebellum indicated that the HiB5 cells predominantly localized to the granular layer. In this site, labeled cells extended short processes that remained confined to the granular layer and a longer, finer process that bifurcated within the molecular layer. Both of these features are characteristic of cerebellar granule neurons in situ (Palay & Chan-Palay 1974). A smaller population of HiB5 cells was found within the molecular layer and displayed processes with a branching pattern reminiscent of Bergmann glia. Although many of the immortal cells were in or near the Purkinje cell layer, none of these cells appeared to differentiate into Purkin je cells. These results, combined with those from the hippocampal transplants, suggest that the survival and/or differentiation of immortalized embryonic precursors is enhanced in regions of active histogenesis. Site-specific factors within the mitotic areas appear to strongly influence the differentiated phenotype of precursors. This was particularly striking in cases in which the hippocampal-derived HiB5 cells engrafted into the cerebellum differentiated into cerebellar-like cell types. Most importantly, the clonal population differentiated into multiple lineages (neurons and glia) in vivo, indicating that exogenous signals play a role in the fate choice of neural precursors.

Immortalized Cerebellar Precursors

The properties of the HiB5 cells after grafting may have been unique to a precursor population isolated from the embyonic hippocampus. However, this possibility is discounted by studies of immortalized precursors derived from the neonatal cerebellum. In this work, mitotic cells obtained from the cerebellar external germinal layer (EGL) of four-day-old mice were immortalized with a retroviral vector carrying the v-myc oncogene (Ryder et al 1990; see above). In contrast to the HiB5 cells, several cerebellar lines established from this infection were multipotent in vitro and generated progeny with neuronal, astrocyte, and oligodendrocyte morphologies and/or antigenic phenotypes. Two of the multipotent clonal populations generated from the mouse cerebellum were infected a second time with a retrovirus containing the lacZ gene to easily identify the lines for in vivo characterizations (Snyder et al 1992). The clones were then implanted into the EGL of the cerebellum of newborn mice and analyzed both during periods of active histogenesis in the cerebellum (spostnatal day 7) and after the development and differentiation of intrinsic cerebellar cells had stopped (1-22 months of age).

Early after grafting (≤1 week), labeled cells assumed a spindle-shaped morphology indicative of cells migrating from the EGL. Subsequently, the grafted cells were distributed in many cerebellar structures and showed site-specific differentiation into several different cell types. For example, cells engrafted within the glial-rich molecular layer displayed an astrocyte morphology and labeling for the astrocyte marker GFAP, whereas the cells that localized within white matter tracks resembled oligodendrocytes. In addition to these glial subtypes, two distinct neuronal populations were observed. Cells engrafted within the internal granular layer possessed a morphology suggestive of granule neurons. This identification was confirmed by electron microscopic analyses of the cells, which revealed small somata, few cytoplasmic organelles, and large nuclei containing condensed chromatin blocks (Figure 2A). At very long periods postimplantation (22 months), some of the engrafted granule neurons established synaptic interactions with endogenous fibers in the host brain (Figure 2B-D). A second population of immortalized cells within the lower molecular layer resembled basket neurons with electron microscopic features such as large somata and indented nuclei containing dispersed, nonaggregated chromatin. The clonal origin of these diverse cell types was con-



Figure 2 Electron micrographs of immortalized cerebellar precursors engrafted within the internal granular layer (IGL) of the cerebellum 22 months postimplantation. (A) Cells expressing the *lacZ* gene are recognized by a dense histochemical precipitate that rings the nucleus. The grafted cell indicated in this view [LGC (labeled granule cell)] displays ultrastructural features, such as condensed chromatin blocks within the nucleus and meager cytoplasm, that are characteristic of endogenous granule cells. (B) Synaptic interactions between engrafted cells and host cells (boxed area in A) shown at higher power. The two blocked regions in B are shown at higher power in C and D to indicate mossy fiber terminals (mf) on precipitate filled (p) dendrites of engrafted granule cells (gd) (arrows) and puncta adherentia (arrowheads) between engrafted and endogenous granule cell dendrites. Reprinted with permission from Snyder et al 1992. Copyright Cell Press.

firmed by inverse polymerase chain reaction performed on tissue dissected from engrafted regions of the cerebellum. These in vivo results were consistent with those obtained with the cerebellar clones in vitro and indicated that a single cerebellar precursor has the potential for generating multilineage progeny within the brain. The cytoarchitecturally appropriate manner in which the grafted cells integrated into the host cerebellum suggests that local environmental signals play a key role in the differentiation of the precursor population. Finally, the multipotency displayed both by the cerebellar cells derived from the neonatal mouse and the HiB5 cells derived from the embryonic rat strongly suggests that such lability is not unique to a particular subset of neural precursors within the CNS.

The marked plasticity of immortalized precursor cells in regions of active histogenesis suggests that the developmental potential of the precursors may be extremely broad if exposed to appropriate environmental signals. This has been confirmed in a study that assessed the fate of immortalized cerebellar precursors implanted into the embryonic CNS (Snyder et al 1993). The cell line was injected into the ventricular system of fetal mice, where cells would have access to multiple regions of the developing CNS, and then examined at adulthood. As observed for immortalized cells grafted into localized areas of the neonatal brain, cerebellar cells implanted into the embryos showed highly site-specific differentiation. For example, cells distributed in the corpus callosum displayed a glial morphology, whereas those localized to the striatum were predominantly neuronal. Most striking were observations that the immortal cells showed a wider range of cellular morphologies than those implanted into neonates that included cell types only born during embyrogenesis, such as pyramidal neurons in the hippocampus and Purkinje cells in the cerebellum. The possibility that signals present during mitosis strongly influence the differentiation of immortal precursors has been further supported by observations that the cerebellar cells are restricted to a glial lineage when implanted into adult mice.

The plasticity of immortalized cells following transplantation to the brain may reflect an unusual property of an immortalized population that may not be characteristic of the primary parent cells. This possibility is discounted by recent preliminary work that explored the developmental features of primary neural precursors exposed to a novel environment. In this study, proliferating cells from the neonatal cerebellum of rats were labeled with [³H]thymidine and implanted into the hippocampus of neonatal rats (Vicario et al 1993). As observed for the HiB5 cells implanted into the developing hippocampus, the primary cerebellar cells localized within the granule cell layer of the dentate gyrus. As observed for granule neurons of the host hippocampus, the engrafted cerebellar-derived cells expressed calbindin (a marker that is not typically expressed by cerebellar cells) and showed upregulation of *c-fos* in response to kainic acid. These results indicate that some primary cells within the newborn brain can differentiate into cell types that are normally beyond their developmental potential when exposed to changes in environmental signals.

Immortalized Medullary Raphe Precursors

Work with immortal precursors suggests that quiescent regions of the CNS may lack or produce few of the signals that promote cellular development. However, there is evidence that the adult CNS expresses environmental signals that can guide the differentiation of neural precursors. This has been shown

in studies of RN33B cells derived from the medullary raphe. As described above, these cells appeared to be neuroblasts because they displayed solely neuronal morphologies upon differentiation in vitro and expressed neuronal markers such as NSE and NF (Whittemore & White 1993).

The RN33B cells were used to explore two issues: first, to assess whether the precursor population would respond to signals in the mature CNS, and second, to determine if diverse target regions of the raphe cells in situ would differentially influence the morphology of the immortal cells as had been observed in vitro (Whittemore & White 1993; see above). For these studies, the RN33B cells were infected a second time with a *lacZ*-containing retrovirus to facilitate in vivo identification (Onifer et al 1993a) and were implanted into the spinal cord and hippocampus of rats (Onifer et al 1993b). Histological examination of grafted regions indicated that the adult CNS supported the survival of the RN33B cells for at least two weeks postimplantation. In both implantation sites, the immortal precursors exhibited a range of morphologies that was often reflective of endogenous cells in the local environment. For example, immortal cells engrafted within the pyramidal layer of the hippocampus displayed oval or pyramidal somata with elaborate apical and basal processes (Figure 3), which are features characteristic of pyramidal neurons in situ. And some cells implanted within the spinal cord resembled the multipolar morphology of spinal cord interneurons.

There were clear differences in the extent to which RN33B cells differentiated in different target regions, however. Most marked was the observation that the majority of the cells engrafted within the spinal cord exhibited a bipolar morphology with very small somata that did not resemble the morphology of any endogenous neurons surrounding the implanted cells. Rather, these RN33B cells were very similar to the undifferentiated morphology of the line in vitro, suggesting that the spinal cord environment did not adequately supply the factors necessary for complete precursor differentiation. In contrast, immortal cells implanted into the hippocampus generally displayed larger somata with more complex process formation. The location of these cells typically correlated with regions of the hippocampus that produce high levels of BDNF, NGF, and NT-3, suggesting a potential role for these neurotrophins in cellular differentiation in vivo. This possibility is supported by the finding of less precursor differentiation within the adult spinal cord, a region that does not synthesize these neurotrophic molecules. Differentiation cannot be solely linked to soluble factors, however, since the RN33B cells often displayed diverse cell types in close proximity to one another. Substrate cues most likely provide an additional guide for the developing neuroblasts. Although the factors that influence precursor development remain to be fully elucidated, these results demonstrate that the adult CNS retains the capacity to direct the differentiation of neural precursors. Furthermore, the microenvironment in



Figure 3 Photomicrograph of *lacZ*-expressing RN33B cells engrafted within the CA3 region of the adult hippocampus 8 weeks postgrafting. Implanted cells displaying β -gal immunoreactivity show extensive apical and distal processes that are characteristic of endogenous cells located in the pyramidal layer of CA3. Micrograph courtesy of LS Shihabuddin, SR Whittemore & VR Holets.

which the precursors develop clearly plays a role in cellular identity, which is consistent with results obtained with immortalized precursors implanted into the immature CNS.

THERAPEUTIC POTENTIAL OF NEURAL PRECURSORS

There has been a great deal of interest in restoring neural function within the damaged CNS through the use of neuronal transplantation (see Fisher & Gage 1993). Most of this work has focused on the use of freshly dissected embryonic neural tissues for grafting, since fetal cells survive and function very well when

introduced into a host CNS. The cultured precursor populations provide a powerful alternative cellular source for transplantation because these cells have the capacity for generating site-specific neural cell types. In the following section, some recent studies that have explored the therapeutic potential of cultured precursor populations are discussed.

Immortalized Neural Cells

The immortalized cerebellar precursors that successfully expressed the lacZ transgene for as long as 22 months (Snyder et al 1992; see above) have been explored for their ability to transport a therapeutic gene into the CNS of mice with a neurovisceral lysosomal storage disease (Snyder et al 1994). The model used to assess gene transfer with neural precursors is the mucopolysaccharidosis type VII mouse, which has a deficiency in the enzyme β -glucuronidase (GUS). This enzyme deficiency results in early death of the animals from lysosomal storage of undegraded glycosaminoglycans in many areas, including the liver, kidney, and CNS (Birkenmeier et al 1989). Two approaches that have been used to try and correct this enzyme deficiency are bone marrow transplantation and somatic gene therapy directed toward bone marrow cells (Birkenmeier et al 1991, Wolfe et al 1992). However, even when substantial improvements in GUS levels are obtained in peripheral organs, there are only minor corrections in the brain (Birkenmeier et al 1991). Thus, the development of cells that can function as enzyme carriers to the brain would provide an important adjunct therapy to peripheral treatments.

The immortalized cerebellar precursors that expressed the *lacZ* transgene (Snyder et al 1992; see above) were further modified to express human GUS (Snyder et al 1994). These cells were then implanted into newborn mucopolysaccharidosis type VII mice by using a technique that resulted in diffuse engraftment throughout the neural axis. Grafted mice were analyzed as adults for transgene expression (*lacZ* and GUS), GUS enzymatic activity, and anatomical changes in lysosomal storage. The modified precursors showed robust engraftment throughout the brain, which was associated with a diffuse elevation of GUS activity to 1-4% of normal levels. Levels of GUS as high as 20% of normal, which is sufficient to have a neuropathologic impact, have been observed in some regions of the grafted brain. Indeed, some areas of the brain have shown a reduction in abnormal lysosomal storage. To date, the modified precursors have been seen to survive and express GUS for at least 8 months postgrafting, suggesting that such a strategy may provide an effective long-term treatment for enzyme deficiencies.

Primary Neural Cells

The bipotent O-2A precursors that generate oligodendrocytes and type 2 astrocytes in vitro (see above) have been explored as a potential source of grafting material to replace myelin in animal models of glial loss (e.g. spinal cord damage) or degeneration (e.g. multiple sclerosis). Demyelinating lesions in adult rats have been achieved by injecting 0.1% ethidium bromide into an area of the spinal cord that was previously subjected to localized irradiation by X rays. Such treatments produce a region of demyelinated axons within a glial-poor environment that prohibits endogenous repair (Crang et al 1992). This damage thus provides a system for assessing the ability of transplanted glial precursors to reconstruct appropriate myelination in the injured zone (for review see Blakemore & Franklin 1991).

The O-2A cells used for grafting are typically derived from the optic nerve of P7 rats. These cells can be expanded and enriched for the precursor population by culturing the cells in the presence of PDGF and bFGF (Bögler et al 1990). When implanted into the damaged spinal cord, O-2A precursors predominantly differentiate into oligodendrocytes while a smaller percentage (1%) appear to generate astrocytes (Crang et al 1992). The graft-derived oligodendrocytes have been seen to remyelinate as much as 90% of the denuded axons in the damaged region. These newly formed myelin sheaths are generally thinner than those found in the intact spinal cord but are otherwise indistinguishable from CNS myelin. The graft-derived origin of the replacement myelin has been confirmed in some cases by premarking the O-2A cells with the lacZ gene prior to transplantation (Crang et al 1991). These results indicate that cultured O-2A cells retain their bipotent nature when reintroduced into the CNS. Furthermore, oligodendrocytes that arise from these implanted progenitors function appropriately, as indicated by the formation of myelin sheaths around axons. Finally, in addition to their ability to repair regions of glial loss, the ability of the O-2A cells to express a transgene after grafting indicates that these precursors can provide a cellular platform for transporting therapeutic gene products into the CNS.

Primary neural populations that are capable of generating both neurons and glia for extended periods in vitro have also been explored as a potential source of neural tissue for CNS transplantation (Gage et al 1994, Ray 1994). Cells obtained from the embyronic hippocampus of rats show proliferative activity in vitro when exposed to high concentrations of bFGF (Ray et al 1993; see above). Differentiated cells that arise in these cultures express several neuronal markers, including NSE, NF, calbindin-D, MAP- 2, MAP-5, and glutamic acid decarboxylase (GAD). These cells, marked with ³H-thymidine for in vivo identification, have been implanted into the hippocampus of adult rats and assessed for cell survival and differentiation for up to 6 weeks post-grafting (Ray 1994). Upon histological analyses, many of the implanted cells were found distributed throughout the dentate gyrus of the hippocampus, where some expressed the granule cell marker calbindin. Other engrafted cells displayed the markers gamma-aminobutyric acid (GABA) or GFAP. These results

indicate that bFGF-expanded populations of cultured embryonic neural cells provide a viable source of neurons in vivo that may be useful for repopulating areas of the CNS that have been damaged. Recently, similar results have been obtained with cells isolated from the hippocampus of adult rats (Gage et al 1994). Moreover, since bFGF enhances the proliferation of neural precursors in vitro, transgenes have been successfully incorporated into the adult hippocampus-derived cells in vitro. These genetically modified cells have been implanted into the adult rat brain where a subgroup of engrafted cells has been seen to express the transgene for at least 6 weeks postimplantation. Thus, in addition to providing a replacement source of neurons and glia for the CNS, cultured neural precursors can be manipulated to carry genes into the brain or spinal cord that may encode for factors that supplement or replace vital molecules.

Finally, the subventricular zone may also provide a source of neuronal cells for transplantation. Lois & Alvarez-Buylla (1994) recently isolated cells from the subventricular zone of transgenic mice that expressed the β -gal gene from a neuron-specific promoter. When implanted into the lateral ventricles of adult hosts, the grafted cells generated progeny that selectively migrated through the adult brain to the olfactory bulb, where they differentiated into two types of olfactory interneurons. Although the engrafted subventricular cells are solely committed to olfactory cell types, exposing the cells to factors such as bFGF may be useful for driving subventricular-derived precursors toward alternative neural fates (see Vescovi et al 1993). The successful manipulation of the migration path and/or lineage decisions of subventricular precursors may offer an additional therapeutic strategy for replacing neuronal populations in the damaged brain.

Neural Precursors In Situ

Overwhelming evidence for a role of growth factors in precursor proliferation and/or differentiation suggests a potential route for activating putative stem cells within the CNS to replace neurons that are lost through damage or disease. Such an intriguing notion was recently pursued by Tao and colleagues (1993); they injected bFGF systemically into neonatal rats to determine if the growth factor could induce neurogenesis in vivo. Mitotic activity was assessed using [³H]thymidine. In comparison to vehicle-injected controls, the bFGF-treated animals showed as much as a 50% increase in [³H]thymidine incorporation in cerebellar tissues. Granule cells isolated from the cerebellum of the bFGFtreated animals by centrifugation appeared to be at least one of the populations that contributed to the proliferative pool. Although quite preliminary, these results highlight the potential for manipulating resident precursor populations within the CNS. Developing methods for successfully provoking selective neurogenesis in the brain will have wide ranging ramifications for repairing neural dysfunction.

SUMMARY

The nervous system of adult mammals, unlike the rest of the organs in the body, has been considered unique in its apparent inability to replace neurons following injury. However, in certain regions of the brain, neurogenesis occurs postnatally and continues through adulthood. The nature, fate, and longevity of cells undergoing proliferation within the CNS are unknown. These cells are increasingly becoming the focus of intense scrutiny; this is a recent development that has led to considerable controversy over the appropriate terminology to describe neural cells as they pass through different stages of proliferation, migration, and differentiation. Continuing studies detailing the properties of mitotic populations in the adult CNS will provide a better understanding of the nature of these cells during their development and should lead to a more consistent nomenclature.

Studies of neural precursors isolated from the embryonic brain have indicated that many subgroups of cells undergo mitosis and subsequent differentiation into neurons and glia in vitro. A number of substances, such as growth factors and substrate molecules, are essential for these processes and also for lineage restriction and fate determination of these cells. Recent studies have shown that cells with proliferative capabilities can also be isolated from the adult brain. The nature of these cells is unknown, but there is evidence that both multipotent cells (stem cells) and lineage-restricted cells (neuroblasts or glioblasts) are resident within the mature CNS and that they can be maintained and induced to divide and differentiate in response to many of the same factors that influence their embryonic counterparts. Presently, it is unclear how many potentially quiescent precursor cells exist in the adult brain or what combination of growth factors and substrate molecules is involved in the proliferation and differentiation of these cells. Some of these questions are currently being addressed by using immortalized neural precursors or growth factor-expanded populations of primary precursors to model precursor responsiveness to environmental manipulations.

Because in vitro culture conditions are unlikely to provide all of the factors necessary for inducing the proliferation and differentiation of neural precursors, recent studies have explored the properties of well-characterized precursor populations after implantation back into specific regions of the developing or adult CNS. These studies have highlighted the importance of the microenvironment in precursor differentiation and further suggested that precursor plasticity is a characteristic that is probably common to neural precursors throughout the CNS. In a therapeutic context, the identification of factors that drive neural precursors toward a desired phenotype will have far-ranging implications for using such cells to repair select regions of the damaged brain.

Ongoing work may show that many areas of the adult CNS that are not known to undergo neurogenesis do so, albeit slowly and with spatial diversity. Furthermore, as the brain matures, many quiescent populations may retain a proliferative capability that can be reactivated. As the factors that control the proliferation and differentiation of these cells in situ are clarified, it may become possible to achieve site-specific cellular replacement in vivo in the diseased or injured CNS.

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