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Amphiregulin Is a Mitogen for Adult Neural Stem Cells

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Neurons are continuously generated from stem cells in the hippocampus and along the lateral ventricles in the adult brain. Neural stem cells can be propagated in vitro in the presence of epidermal growth factor (EGF) or fibroblast growth factor-2. We report here that amphiregulin, a growth factor related to EGF, is a mitogen for adult mouse neural stem cells in vitro and displays potency similar to that of EGF. Neural stem cell cultures can be initiated and the cells propagated as efficiently in the presence of amphiregulin only as with EGF. Furthermore, we show that amphiregulin is expressed in the choroid plexus of the ventricular system and in the hippocampus in the adult brain, suggesting that amphiregulin may participate in the regulation of neural stem cell proliferation and neurogenesis in the adult brain.

Key words: EGF; neurogenesis; proliferation; differentiation

Neural stem cells in the adult mammalian brain continuously generate new neurons, predominantly in the hippocampus and olfactory bulb (McKay, 1997; Momma et al., 1999; Temple and Alvarez-Buylla, 1999; Gage, 2000; van der Kooy and Weiss, 2000; Gould and Gross, 2002; Rakic, 2002). The existence of neural stem cells in the adult brain was first demonstrated by the expansion of self-renewing multipotent cells in the presence of epidermal growth factor (EGF; Reynolds and Weiss, 1992, 1996). Neural stem cells can also be propagated in the presence of fibroblast growth factor (FGF)-2 (Richards et al., 1992; Palmer et al., 1995; Gritti et al., 1996).

The EGF family consists of six structurally related proteins: EGF, transforming growth factor-α (TGF-α), amphiregulin, heparin-binding EGF, betacellulin, and epiregulin, which all bind and activate the EGF receptor ErbB1 (Yarden and Sliwkowski, 2001). These proteins have structural similarities in that they all have what is known as the *EGF-like motif*. EGF has well-characterized effects on embryonic and adult neural stem cells in vitro. Abnormal brain development in ErbB1 null mice points to the importance of this signaling pathway in neural development (Sibilia et al., 1998). However, the physiological role of EGF and related growth factors in adult neurogenesis is poorly understood. EGF is expressed in ventrocaudal parts of the adult brain, which are not in close proximity to the neurogenic regions (Fallon et al., 1984). TGF-α is expressed in the adult striatum and olfactory bulb (Wilcox and Derynck, 1988; Seroogy et al., 1993). TGF-α null mice show reduced cell proliferation in the lateral ventricle wall and a reduced number of newborn neurons in the adult olfactory bulb, demonstrating a physiological role for TGF-α in this process (Tropepe et al., 1997). However, analysis of mice with targeted deletions of EGF and its related ligands has revealed specific and overlapping functions for these molecules, and the milder phenotype of TGF-α than ErbB1 null mice (Tropepe et al., 1997; Sibilia et al., 1998) suggests that several EGF-related molecules may regulate neural stem cell proliferation and neurogenesis in the adult brain.

We demonstrate here that amphiregulin is a mitogen for adult neural stem cells as potent as EGF. Moreover, we show that amphiregulin is highly expressed by cells in the choroid plexus as well as in cells in the hippocampus, suggesting that amphiregulin may regulate neural stem cell proliferation and neurogenesis in the adult brain.

MATERIALS AND METHODS

Neural Stem Cell Cultures

The lateral walls of the lateral ventricles of adult female C57/Bl mice (Charles River) were dissected and enzymatically dissociated in 0.5 μg/μl trypsin (Sigma, St. Louis, MO) and 0.7 μg/μl hyaluronidase (Sigma) in phosphate-buffered saline (PBS) at 37°C for 30 min. The cells were then processed as described elsewhere (Johansson et al., 1999) and cultured in DMEM/F12 supplemented with B27 (Life Technologies, Bethesda, MD), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) and either the growth factor EGF (20 ng/ml; BD Bioscience) or amphiregulin (20 ng/ml; R & D Systems, Minneapolis, MN) or both. In each well, 37,500 cells

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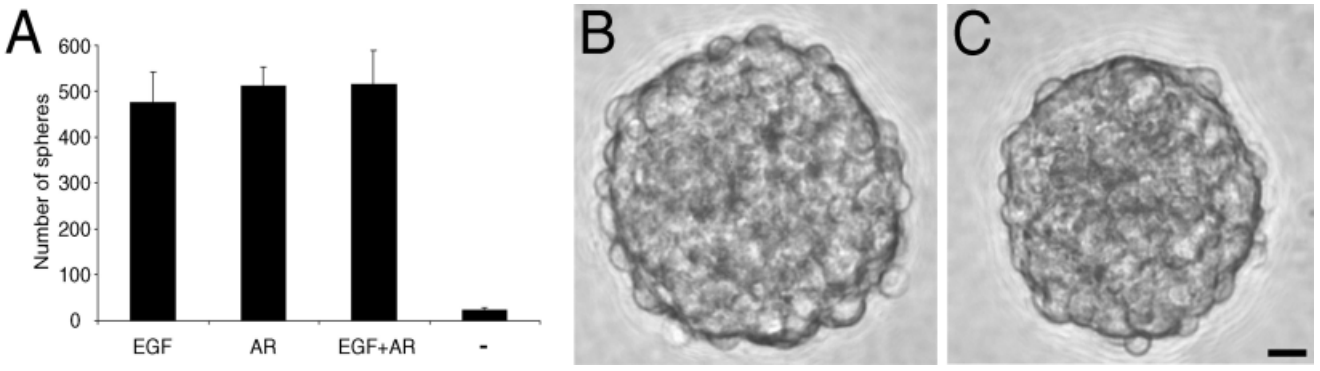

Fig. 1. Neural stem cells can be propagated in the presence of amphiregulin. Neural stem cells isolated from the lateral ventricle of adult mice were cultured in vitro in the presence of EGF, amphiregulin (AR), EGF and amphiregulin, or without growth factor (–). The total numbers of neurospheres were counted after 5 days in vitro. Bars represent the mean number of spheres ± SEM from five independent experiments (**A**). In each independent experiment, 150,000 lateral ventricle wall cells from two animals were divided between the four different conditions, and each bar thus represents the average number of spheres derived from one lateral ventricle. The neurospheres cultured in EGF (**B**) or amphiregulin (**C**) had indistinguishable morphology and were of comparable size. Scale bar = 10 μm.

were plated in 2 ml medium. Growth factors were added to the cultures every second day. The total number of neurospheres was counted 5 days after the cultures were initiated. Three days later, the neurospheres were dissociated by incubation at 37°C for 5 min with trypsin-EDTA (0.5 μg/μl; Life Technologies). The total number of secondary neurospheres was counted 5 days after the dissociation. The average of the total number of spheres from five independent experiments in the different culture conditions was calculated.

Differentiation and Immunocytochemistry of Neural Stem Cells

Five-day-old primary or secondary neurospheres were transferred to poly-L-ornithine (15 μg/ml; Sigma)- and laminin (4 μg/ml; Life Technologies)-coated coverslips. The spheres were allowed to attach and differentiate for 2 days before they were fixed in 4% formaldehyde in PBS for 15 min at room temperature. To study the differentiation potential of the neural stem cells grown in the presence of the different growth factors, differentiated cells were triply stained with antibodies against cell type-specific epitopes: neurons (mouse anti-βIII-tubulin antibody, Tuj1, 0.7 μg/ml; Babco), astrocytes (mouse anti-GFAP antibody, 2 μg/ml; Sigma), and oligodendrocytes (mouse anti-O4 11, μg/ml; Chemicon, Temecula, CA) for 1 hr at room temperature. The primary antibodies were detected with Cy3-conjugated goat anti-mouse antiserum (2.8 μg/ml; Jackson Immunoresearch, West Grove, PA), Cy2-conjugated goat anti-mouse antiserum (6 μg/ml; Jackson Immunoresearch), and AMCA-conjugated goat anti-rabbit antiserum (75 μg/ml; Jackson Immunoresearch). All antibodies were diluted in 4 mg/ml bovine serum albumin (BSA; Sigma) and 0.1% saponin (Sigma) in PBS. The coverslips were mounted on object slides using n-propyl gallate (Sigma) in glycerol.

Immunohistochemistry

Adult female C57/Bl mice (Charles River) were perfused with 4% formaldehyde in PBS, and the brain was removed and postfixed in 4% formaldehyde in PBS at 4°C for 2 hr. Before the brain was cryostat sectioned (14 μm), it was incubated in 20% sucrose at 4°C overnight. To localize amphiregulin in the brain, the slides were washed three times in PBS and blocked in 10% normal goat serum (Dako, Carpinteria, CA) or normal donkey serum (Jackson Immunoresearch) diluted in PBS, 5% BSA (Sigma), and 0.3% Triton X-100 (Sigma) for 30 min at room temperature. Amphiregulin was detected with either a mouse antiamphiregulin antibody (7.5 μg/ml; R & D Systems) or a goat antiamphiregulin antibody (7.5 μg/ml; R & D Systems), which were incubated overnight at 4°C. After several washes in PBS, the primary antibodies were detected with Cy3-conjugated goat anti-mouse (1.4 μg/ml; Jackson Immunoresearch), Cy3-conjugated donkey anti-mouse (1.4 μg/ml; Jackson Immunoresearch), biotin-conjugated rabbit anti-goat (1:200; Sigma), or biotin-conjugated horse anti-mouse (1:200; Vector Laboratories, Burlingame, CA) antiserum. Biotin-conjugated antibodies were visualized using the Vectastain ABC kit with diaminobenzidine (DAB; Vector Laboratories). Cell nuclei were labeled with DAPI (1.5 μg/ml; Sigma), and the slides were mounted with Vectashield (Vector Laboratories).

RESULTS

We first examined whether adult mouse neural stem cells can be propagated in the presence of amphiregulin as the only mitogen. Neural stem cell cultures were established from the lateral walls of the lateral ventricles of adult mice under conditions in which they form clonal spheroid colonies, referred to as *neurospheres* (Reynolds and Weiss, 1992). The neural stem cells were cultured under four different conditions: in the presence of EGF, amphiregulin, EGF and amphiregulin, or without growth factor. The total number of neurospheres in the four different culture conditions was counted after 5 days in vitro, and the average number of neurospheres from five independent experiments was calculated (Fig. 1A). In each independent experiment, 150,000 lateral ventricle wall cells from two animals were divided between the four different conditions. No neurospheres or some very small spheres were

detected in the cultures without supplemented growth factor. The number and the morphology of the neurospheres were indistinguishable in the cultures grown in the presence of amphiregulin, EGF, or amphiregulin and EGF (Fig. 1).

We next tested whether the neurosphere-forming cells under the different conditions had self-renewal capacity and whether cells expanded under one condition could be propagated in another mitogen. The neurospheres were dissociated after 8 days in vitro, and each culture grown in a single growth factor was divided into two halves; one half continued to grow in the same growth factor as before the passage, and the other half was grown in the other growth factor. The neurospheres grown in both growth factors continued to grow in both growth factors, and the neurospheres grown without growth factor continued without growth factor. Five days after the passage, the total number of neurospheres was counted from five independent experiments. On average, 12 secondary spheres were generated from each primary neurosphere, and there was no significant difference between the different culture conditions in size or number of neurospheres, except for those cultured in the absence of mitogen, where no secondary spheres formed (Table I).

These results show that adult neural stem cells can be propagated in the presence of amphiregulin and that amphiregulin can fully substitute for EGF. Moreover, the fact that the neural stem cells initially expanded in one of the growth factors can be propagated in the presence of the other growth factor suggests that the same cell type is responding to EGF and amphiregulin.

TABLE I. Number of Secondary Neurospheres Generated in Different Mitogens From Five Independent Experiments

Mitogen	Number of neurospheres (average ± SEM)
EGF + AR	2,645.8 ± 1,052
EGF → EGF	3,934 ± 2,340
EGF → AR	3,046 ± 1,930
AR → AR	2,298 ± 1,169
AR → EGF	1,992 ± 902
No mitogen	17 ± 12

To investigate whether the neural stem cells grown in the different growth factors have the same potential to differentiate into all the three main neural cell types, i.e., neurons, astrocytes, and oligodendrocytes, we induced differentiation by plating primary neurospheres on an adhesive substrate. After 2 days of differentiation, the cells were triply labeled with antibodies against early markers for neurons (βIII-tubulin), astrocytes (glial fibrillary acidic protein; GFAP), and oligodendrocytes (O4). All cultures, independently of culture conditions, produced all three cell types (Fig. 2A–C). The same result was observed with secondary neurospheres (data not shown). The differentiation potential of the amphiregulin-responsive adult neural stem cells thus appears to be the same as that for the EGF-responsive neural stem cells.

We went on to study whether amphiregulin is expressed in the brain. Sections from the adult mouse brain were analyzed with two different antibodies to amphiregulin. Prominent amphiregulin immunoreactivity was seen in the meninges and in perivascular cells lining blood vessels. Throughout the brain, scattered cells with a stellar morphology displayed weak amphiregulin immunoreactivity (data not shown). Stem cells can be propagated primarily from the hippocampus and the walls of the lateral ventricles, and this is where most neurons are born in the adult brain. We therefore specifically analyzed whether amphiregulin is expressed in these regions. No obvious amphiregulin immunoreactivity was seen in the walls of the lateral ventricle, but cells in the choroid plexus showed very prominent amphiregulin immunoreactivity (Fig. 3A,B). The strongest amphiregulin immunoreactivity was seen in the stromal cells of the choroid plexus, although weaker labeling also could be seen in the epithelial compartment (Fig. 3C). The prominent expression in the choroid plexus suggests that amphiregulin may be secreted into the cerebrospinal fluid and in this way may reach neural stem cells lining the ventricular system. In

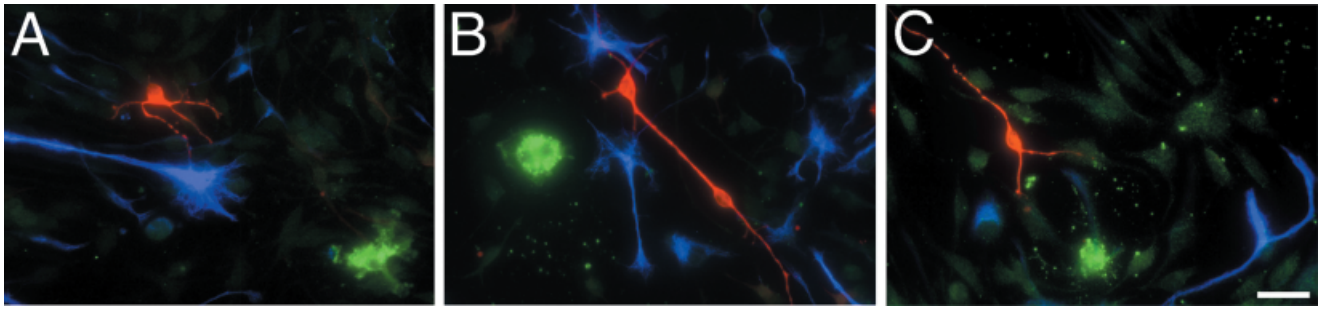

Fig. 2. Differentiation potential of neural stem cells cultured in different mitogens. Neural stem cells from the lateral ventricle of adult mice were propagated in vitro for 5 days to generate neurospheres in EGF (**A**), amphiregulin (**B**), or EGF and amphiregulin (**C**) and then induced to differentiate by plating on poly-L-ornithine- and laminin-coated coverslips for 2 days. Neural stem cells cultured under any of the growth conditions differentiated to all three neural cell types as revealed by immunocytochemical detection of early markers for neurons (βIII-tubulin; red), astrocytes (GFAP; blue), and oligodendrocytes (O4; green). Scale bar = 20 μm.

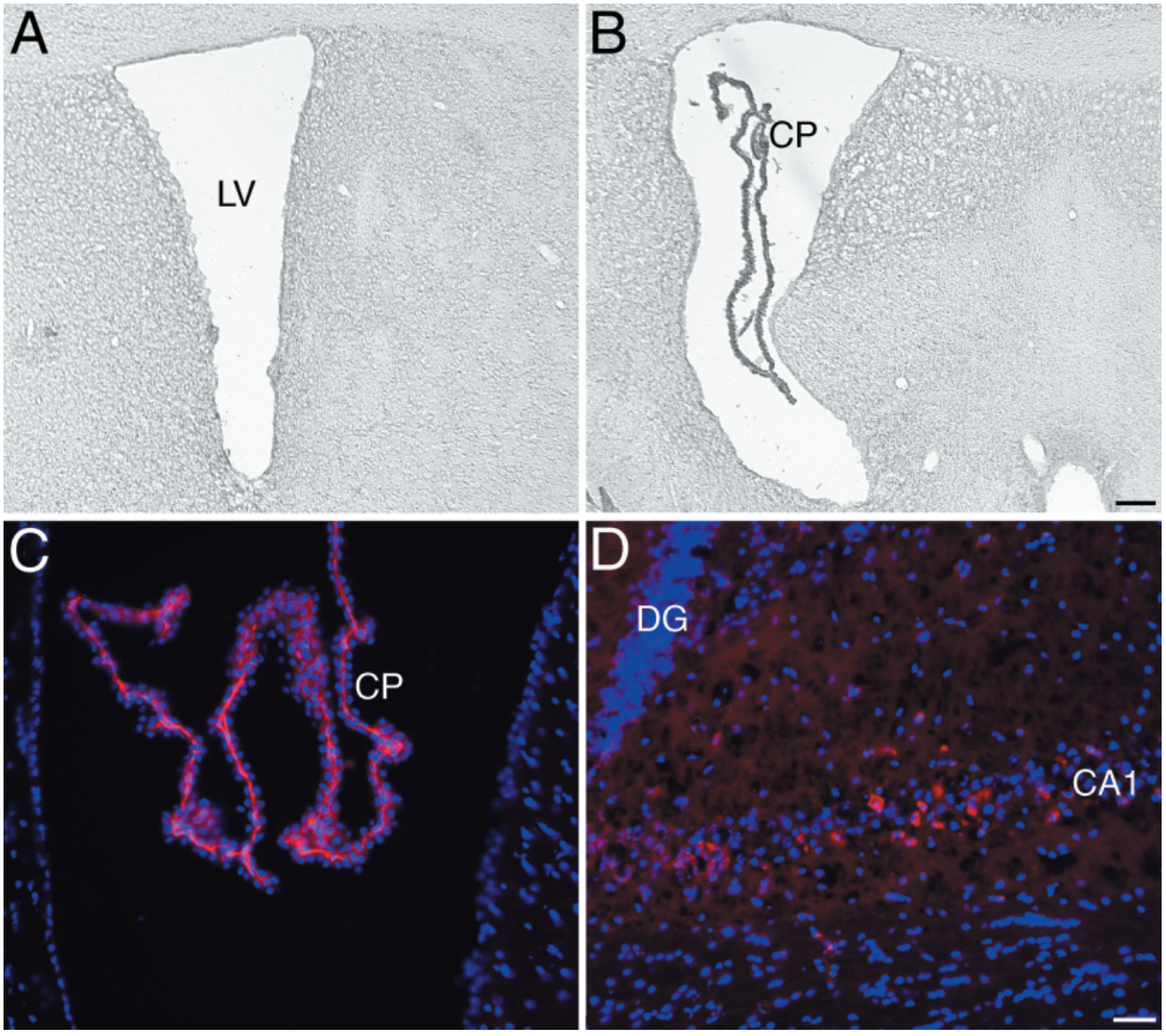

Fig. 3. Amphiregulin expression in the adult mouse brain. Amphiregulin was detected in the adult mouse brain with two different antibodies, goat antiamphiregulin (**A,B**) and mouse antiamphiregulin (**C,D**). Neural stem cells are abundant in the walls of the lateral ventricles (LV). Serial sections did not reveal any amphiregulin immunoreactivity in the anterior part of the lateral ventricle (A), but very prominent labeling in the choroid plexus (CP), which is located more posteriorly (B). The amphiregulin immunoreactivity (red) was strongest in the stromal cells of the choroid plexus, but much weaker labeling could also be detected in the epithelial cells (C). The hippocampus is another neurogenic region, and scattered amphiregulin-immunoreactive cells could be seen in all major subdivisions of the hippocampus. In D, amphiregulin-immunoreactive (red) cells with neuronal morphology are seen in the pyramidal layer of CA1 (CA1). The dentate gyrus is labeled (DG). Cell nuclei in C and D are labeled with DAPI and appear blue. Scale bar = 100 μm in B (for A,B); 40 μm in D (for C,D).

addition to amphiregulin immunoreactivity in scattered cells with a stellar shape, neurons located in the pyramidal layer of the CA1/subicular border region of hippocampus displayed prominent amphiregulin immunoreactivity (Fig. 3D). Thus, amphiregulin is expressed in the two major neurogenic regions of the adult mouse brain and may have a physiological role in stem cell proliferation and neurogenesis.

DISCUSSION

We have here demonstrated that adult neural stem cells can be propagated in vitro in the presence of amphiregulin, without the need for other mitogens. The neural stem cell cultures could be initiated with amphiregulin as the only growth factor, demonstrating that amphiregulin responsiveness was not aquired as a result of long-term

culturing but, rather, suggesting that at least some neural stem cells are responsive to amphiregulin in vivo. The fact that the neurosphere cells could be passaged to form new neurospheres that were multipotent demonstrates that the amphiregulin-propagated cells are bona fide neural stem cells.

Both EGF and amphiregulin can signal through the ErbB1 tyrosine kinase receptor (Yarden and Sliwkowski, 2001), implying that it is the same cells from the adult brain that can be propagated in vitro in the presence of either mitogen. This is also supported by the fact that cultures initiated in the presence of one of the growth factors could be propagated in the presence of the other mitogen. Moreover, the lack of synergistic effect of EGF and amphiregulin on cell proliferation (Fig. 1A) further supports that it is the same cell type and the same signaling pathway through which both ligands act.

Does amphiregulin regulate the proliferation of neural stem cells in vivo? The prominent expression of amphiregulin in the choroid plexus implies that this mitogen may be released to the cerebrospinal fluid and may reach neural stem cells along the ventricular system. ErbB1, a receptor for amphiregulin, is expressed in cells in the walls of the lateral ventricle (Seroogy et al., 1995). That ErbB1 ligands indeed can reach and affect stem or progenitor cells in the lateral ventricle wall through the cerebrospinal fluid is clear, insofar as infusion of EGF into the cerebrospinal fluid results in prominent stimulation of cell proliferation in cells lining the ventricle (Craig et al., 1996; Kuhn et al., 1997; Kojima and Tator, 2000). However, it is at present unclear whether amphiregulin synthesis in the choroid plexus results in concentrations in the cerebrospinal fluid sufficient to affect cells in the walls of the ventricular system. Neural stem cells have been localized both to the ependymal layer and to the subventricular zone (Doetsch et al., 1999; Johansson et al., 1999; Rietze et al., 2001), although it remains unclear whether they represent independent stem cell populations or whether they represent different stages in the same lineage (Momma et al., 1999). EGF infusion in the lateral ventricle results in proliferation of both ependymal and subependymal cells (Craig et al., 1996; Kuhn et al., 1997; Kojima and Tator, 2000).

One could envisage utilizing neural stem cells for therapies to replace lost neurons in neurological diseases in two conceptually different ways, either by expanding the stem cells in vitro to transplant cells or by stimulating the endogenous neurogenesis in vivo. In the first case, it is important to develop optimal expansion protocols for neural stem cells. In the latter situation, there are several steps that may be important to control, for example, stem cell proliferation and the survival of newborn neurons. It is clear that merely stimulating neural stem cell proliferation may not result in an increased number of new neurons, in that infusion of EGF, although it potently stimulates cell proliferation in the ventricle wall, does not yield more neurons (Kuhn et al., 1997). Instead, there is an increased number of new astrocytes in such animals. Infusion of FGF-2, which also is a potent mitogen for neural stem cells, on the other hand, results in increased neurogenesis in the adult brain (Kuhn et al., 1997). Several studies have identified factors that can potently influence the generation of distinct cell types from neural stem cell in vitro (see, e.g., Johe et al., 1996; Caldwell et al., 2001). It will be important to study the potential of different growth factors and cytokines to stimulate neural stem cell proliferation and differentiation in vitro and in vivo in the development of stem cell-based therapies.

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