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Identification of a Neural Stem Cell in the Adult Mammalian Central Nervous System

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Summary

New neurons are continuously added in specific regions of the adult mammalian central nervous system. These neurons are derived from multipotent stem cells whose identity has been enigmatic. In this work, we present evidence that ependymal cells are neural stem cells. Ependymal cells give rise to a rapidly proliferating cell type that generates neurons that migrate to the olfactory bulb. In response to spinal cord injury, ependymal cell proliferation increases dramatically to generate migratory cells that differentiate to astrocytes and participate in scar formation. These data demonstrate that ependymal cells are neural stem cells and identify a novel process in the response to central nervous system injury.

Introduction

The vast majority of cells in the nervous system are born during the embryonic and early postnatal period, but new neurons are continuously added in certain regions of the adult mammalian brain (Altman and Das, 1965). These neurons are thought to derive from a population of stem cells, and it was shown by Reynolds and Weiss (1992) that neural stem cells taken from the adult brain can be propagated in vitro. These cells have the capacity for self-renewal and can generate the major classes of central nervous system cell types, that is, neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss, 1996). Neural stem cells can be isolated from the walls of the ventricular system of the adult central nervous system as well as from the hippocampus (Lois and Alvarez-Buylla, 1993; Morshead et al., 1994; Weiss et al., 1996; Palmer et al., 1997). The stem cells located in association with the lateral ventricles of the brain give rise to immature neurons that migrate along the rostral migratory stream to the olfactory bulb, where they differentiate and integrate as interneurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994; see Figure 1A).

The wall of the ventricular system is composed of a single layer of ependymal cells facing the lumen and a subventricular zone lying beneath the ependymal layer (Figure 1A). The subventricular zone contains three distinct cell types: astrocytes, immature neurons, and a rapidly proliferating cell type (Doetsch et al., 1997). The stem cell has previously been suggested to reside in the subventricular zone (Lois and Alvarez-Buylla, 1993; Morshead et al., 1994). However, there are reasons to believe that the subventricular zone instead harbors the transit amplifying progenitor cell. First, depletion of the rapidly proliferating cell population does not affect the number of stem cells that can be isolated from the brain (Morshead et al., 1994). Second, it is possible to isolate stem cells from all regions of the nervous system containing extensions of the ventricular system, including the spinal cord (Weiss et al., 1996), which does not contain a subventricular zone.

Assuming that the subventricular zone is the site of the transit amplifying progenitor cells, it leaves open the possibility that the cells in the ependymal layer are the adult neural stem cells. This may appear counterintuitive given that ependymal cells are thought not to proliferate in the adult animal and to play a critical role as a barrier between the cerebrospinal fluid and the neural tissue (Del Bigio, 1995). During embryogenesis, however, neural stem cells in the ventricular zone line the lumen of the neural tube, corresponding to the localization of ependymal cells in the adult animal. In addition, nestin, a protein expressed by neural stem cells (Lendahl et al., 1990), is most highly expressed in ependymal cells and at lower levels in the rapidly proliferating subventricular zone progenitor cells in adult mammals (Doetsch et al., 1997). Interestingly, in response to spinal cord injury, nestin expression is increased in ependymal cells, and with time, increasing numbers of nestin-positive cells can be seen outside the ependymal layer, suggesting that new cells may be generated from these cells in response to injury (Frisén et al., 1995).

In this study, we demonstrate that ependymal cells are neural stem cells and provide novel insights into how stem cells participate in the response to central nervous system injury.

Results

Olfactory Bulb Neurons Derive from Ependymal Cells

It is well established that some olfactory bulb neurons are regenerated from cells originating in association with the lateral ventricles and that migrate along the rostral migratory stream (Goldman and Luskin, 1998). To analyze the involvement of ependymal cells in this process, we first devised a method to specifically label the ependymal layer throughout the neuraxis (Figure 1B). A single injection of the fluorescent label DiI resulted in specific labeling of the ependymal layer throughout the ventricular system of adult rats (Figures 1C–1E). All subsequent analyses were done on the hemisphere contralateral to the injection to exclude the possibility of labeling other cell types along the injection route. The initial restriction of the DiI exclusively to the ependymal layer changed

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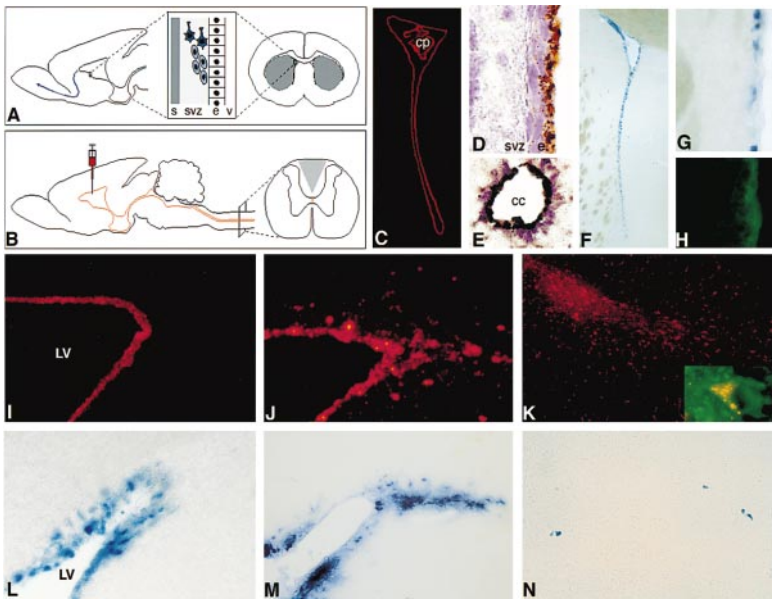

Figure 1. Generation of Olfactory Bulb Neurons from Ependymal Cells

Schematic drawing of the migration of neurons in the adult forebrain and the structure of the wall of the lateral ventricle (A). The ventricle (v) is lined by ependymal cells (e). Between the ependymal layer and the striatum (s) is the subventricular zone (svz) where progenitor cells (light blue) divide to give rise to immature neurons (dark blue). The neurons migrate to the olfactory bulb (blue arrow). (B) Labeling of ependymal cells. DiI- or replication-deficient adenovirus carrying the *lacZ* reporter gene is injected stereotaxically into a lateral ventricle, resulting in labeling of ependymal cells throughout the ventricular system. In some of these animals, an incision (gray area in the spinal cord cross section) was made in the spinal cord dorsal funiculus. The DiI injection labels the ependymal layer lining the lateral ventricle (C and D) and the spinal cord central canal (E) 6 hr after the injection. In (D) and (E), DiI was photoconverted to a brown product and the section was counterstained with Harris stain. The choroid plexus (cp) is indicated in (C), and the central canal (cc) in (E) *lacZ* expression is restricted to ependymal cells 1 day after intraventricular injection of the adenovirus (F and G). The adenovirus receptor CXADR is exclusively localized to ependymal cells within the lateral ventricle wall (H). One day after an intraventricular injection of DiI (I) or adenovirus (L), the labels are restricted to the ependymal layer in the anterodorsal tip of the lateral ventricle. In the same region, labeled cells are seen in the subventricular zone 10 days after injection of DiI (J) or adenovirus (M) into the contralateral lateral ventricle. In these animals, labeled cells are also seen in the olfactory bulb (K and N). The inset in (K) shows immunoreactivity for neuron-specific βIII-tubulin (green) in a DiI-labeled cell in the olfactory bulb. The lumen of the lateral ventricle (LV) is indicated in (I) and (L).

over time with an increasing number of DiI-labeled cells appearing in the subventricular zone a few days after the injection (Figure 1J). Soon after, an increasing number of labeled cells was observed in the rostral migratory stream, and within 10 days the first DiI-labeled cells were seen in the olfactory bulb (Figure 1K). The DiI-labeled cells in the olfactory bulb were immunoreactive for the neuron-specific proteins βIII-tubulin (inset in Figure 1K) and Map2 (data not shown). The same results were obtained when these experiments were performed in mice (data not shown).

As the results critically depend on the unique DiI labeling of ependymal cells, but not of cells in the subventricular zone, the possibility of DiI transfer to the subventricular cells had to be ruled out. To exclude direct cell-to-cell transfer of DiI from the ependymal cells, we cocultured DiI-labeled ependymal cells with genetically labeled cells from ROSA-26 mice. No detectable transfer of DiI to the ROSA-26 cells could be observed. To rule out the possibility of passive DiI diffusion from the cerebrospinal fluid to subventricular cells over time, we aspirated cerebrospinal fluid from the lateral ventricle from animals that had received an intraventricular DiI injection the day before and added it to cultured cells. We found only weak labeling, indicating that the DiI concentration was very low. Moreover, injection of the cerebrospinal fluid into the lateral ventricle of another animal did not result in any labeling of cells lining the ventricle (data not shown). These data suggest that the DiI concentration in the lateral ventricle drops fast after an intraventricular injection and that a delayed passive labeling of cells in the subventricular zone is very unlikely.

We next wanted to specifically label the ependymal cells by an alternative method to confirm the results obtained after DiI injection. We therefore injected a replication-deficient adenovirus expressing *lacZ* into the lateral ventricle of adult rats. One day after injection, *lacZ* expression was exclusively confined to ependymal cells and no labeling was seen in the subventricular zone (Figures 1F and 1G), in keeping with previous observations (see Davidson and Bohn, 1997 and references therein). To analyze the molecular basis for this specific infection of ependymal cells, we studied the distribution of the recently identified adenovirus receptor CXADR (previously known as CAR) (Tomko et al., 1997). Except for scattered cell processes in the brain parenchyma, CXADR immunoreactivity was exclusive to the cell membranes of ependymal cells (Figure 1H). *lacZ* expression was strong in ependymal cells lining the lateral ventricles. In the lateral wall of the lateral ventricle, an increasing number of *lacZ*-expressing cells was seen in the subventricular zone over time (Figure 1M). Similar to the DiI-labeling experiments, *lacZ*-expressing cells were found in the olfactory bulb 10 days postinjection, albeit in much lower numbers (Figure 1N). The lower number of *lacZ*-labeled cells was expected, since the adenovirus is replication deficient and the label is, in contrast to DiI, inherited by only a subset of the progeny of the infected cell. Taken together, the DiI and adenovirus experiments show that a label uniquely restricted to cells in the ependymal layer is later found in cells migrating in the rostral migratory stream and in neurons present in the olfactory bulb.

Ependymal Cells Are Neural Stem Cells

In the presence of mitogens such as EGF or FGF, multipotent neural stem cells can be propagated from

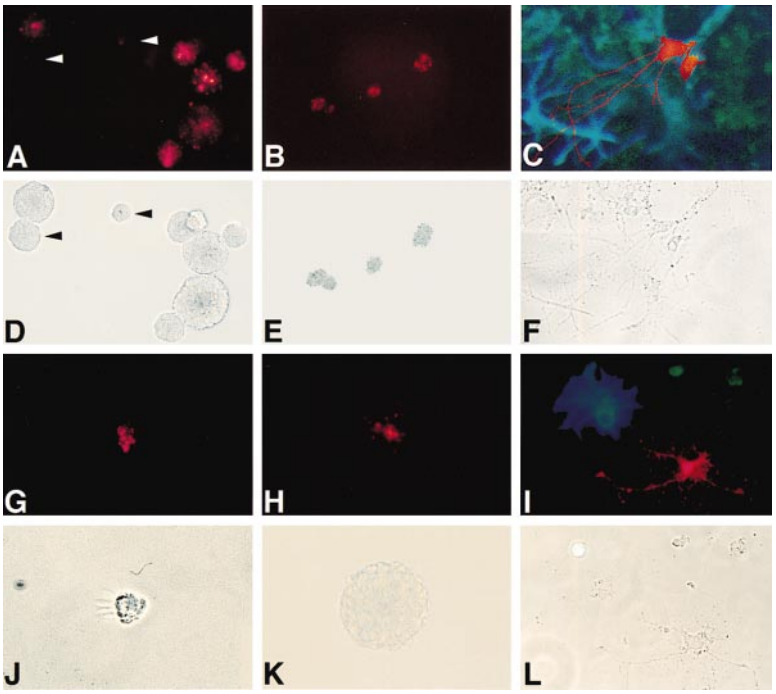

Figure 2. Ependymal Cells Generate Neurospheres and Differentiated Progeny In Vitro

Neurospheres from the brain (A and D) or the spinal cord (B and E) of an animal that had received an intraventricular DiI injection 6 hr prior to the culture. Weakly labeled or unlabeled neurospheres are indicated with arrowheads in (A) and (D). Immunofluorescence localization (C) of βIII-tubulin (neurons, red), GFAP (astrocytes, blue), and O4 (oligodendrocytes, green) in a differentiated secondary sphere derived from a DiI-labeled primary sphere. (F) shows phase contrast of the same field. In other cultures, DiI-labeled ciliated ependymal cells were picked and cultured as single cells in microwells (G and J). These cells formed neurospheres (H and K), and when allowed to differentiate, neurons, astrocytes, and oligodendrocytes were formed from a single sphere (I and L; cell type–specific markers as in [C]).

dissociated adult brain and spinal cord, and single cells form a characteristic spheroid cell aggregate of tightly clustered cells referred to as a neurosphere, where all cells are derived clonally from a single cell (Reynolds and Weiss, 1992, 1996). To test whether ependymal cells from the lateral ventricle and spinal cord are neural stem cells, we cultured dissociated cells from the walls of the lateral ventricle and spinal cord of animals that had received a DiI injection 6 hr earlier. Only the lateral ventricle contralateral to the DiI injection was used. In the initial cell population after dissociation of the lateral ventricle wall tissue, 8.2% ± 0.4% of the cells were DiI labeled (mean ± SEM from three independent experiments). When culturing cells at clonal density using the same conditions previously found to support the growth of adult neural stem cells (Reynolds and Weiss, 1992; Svendsen et al., 1995; Gritti et al., 1996; Weiss et al., 1996), typical spheres of undifferentiated cells formed in the cultures. Examination of these spheres showed that 88.6% ± 1.20% and 89.0% ± 1.23% from lateral ventricle or spinal cord, respectively, were clearly DiI labeled (mean from five independent experiments ± SEM) (Figures 2A–2E). To test the ability of these sphere-forming cells to self-renew, the DiI-labeled spheres were collected and dissociated to single cells. Many of these cells formed new spheres and generated neurons, astrocytes, and oligodendrocytes when induced to differentiate by transferring them to an adhesive substrate (Figures 2C and 2F). As previously described, the frequency of different cell types varied, depending on the culture conditions (Johe et al., 1996). These experiments demonstrate that ependymal cells have the capacity for self-renewal and are multipotent, thus meeting the criteria for being stem cells.

A direct test of the potential of ependymal cells to form neurospheres would be to culture individual ependymal cells in vitro. To this end, we isolated ependymal cells from dissociated lateral ventricle wall tissue by two criteria. First, the cells had to possess cilia, a distinct morphological characteristic of ependymal cells. Second, the tissue was taken from animals that had received a DiI injection 6 hr before sacrifice, and only DiI-labeled cells were collected. Cells that fulfilled both criteria were picked and transferred to microwells (Figures 2G and 2J). One cell was cultured per well in neurosphere-conditioned medium. In these cultures, 58% (111/192) of the ependymal cells underwent cell division. In the majority of these wells (99 wells), the cells died within a few days or formed very small cell clusters. However, 6.2% of the initial cells (12/192) formed large neurospheres (Figures 2H and 2K). When serum was added to the medium of these neurospheres, cells expressing cell type–specific markers for neurons, astrocytes, and oligodendrocytes were identified (Figures 2I and 2L). This revealed that single ependymal cells are capable of forming neurospheres that can generate neurons, astrocytes, and oligodendrocytes, that is, they are bona fide neural stem cells.

Isolation of Neural Stem Cells by Purification of Cells Expressing an Ependymal Cell–Specific Marker

To further test the ability of ependymal cells to form neurospheres, we wanted to complement the DiI experiments described above by isolating ependymal cells in a conceptually different way that would not depend on the labeling of cells in vivo. We reasoned that the transmembrane Notch 1 receptor could be used for immunosorting, since *Notch 1* mRNA is localized in ependymal cells but not in other cells associated with the ventricular wall (Weinmaster et al., 1992). Immunohistochemistry using two different antibodies against Notch 1 extracellular (Mitsiadis et al., 1995) and intracellular (Zagouras et al., 1995) domains produced the same pattern of

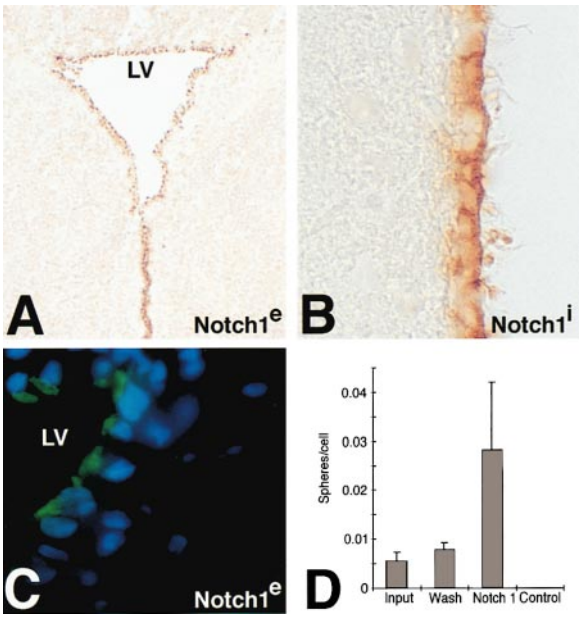

Figure 3. Enrichment of Neural Stem Cells Expressing Notch 1

Immunohistochemical localization of Notch 1 in the wall of the lateral ventricle (A–C) with an antiserum against the extracellular domain (A and C; green in C) or a monoclonal antibody against the intracellular domain (B) of the receptor. Cell nuclei are labeled with DAPI in (C). Dissociated lateral ventricle wall cells were incubated with the antiserum against the ectodomain of Notch 1, followed by incubation with magnetic bead–conjugated secondary antibodies and magnetic separation of labeled (Notch 1 fraction) and unlabeled cells (wash fraction). In control experiments, the primary antiserum was omitted. The number of cells at the start of the culture and the number of neurospheres present 1 week later were counted in each fraction. (D) The number of neurospheres that had formed in each fraction in relation to the number of cells at the start of the culture was then calculated (mean from four independent experiments; error bars show SEM).

staining and showed that only ependymal cells were immunoreactive (Figures 3A–3C). Interestingly, the Notch 1 immunoreactivity, using both antisera, was asymmetrically distributed, such that Notch 1 immunoreactivity was strong in the luminal cell membrane but weak or undetectable in the cell membrane that faced the subventricular zone (Figures 3B and 3C). Notch 1 immunoreactivity was previously found to be asymmetric also in cells of the developing ferret cortex, but in this case Notch 1 protein was predominantly localized on the part of the cell facing away from the lumen of the ventricle (Chenn and McConnell, 1995).

We took advantage of the selective expression of Notch 1 to isolate ependymal cells from acutely dissociated lateral ventricle wall tissue by magnetic sorting with the antiserum against the extracellular domain of Notch 1. The Notch 1 sorted fraction contained 1.77% ± 0.69% (mean from three independent experiments ± SEM) of the dissociated cells from the lateral ventricle wall. Immunolabeling revealed that 95% and 17% of the cells in the Notch 1 sorted and remaining fraction, respectively, displayed Notch 1 immunoreactivity (mean from two independent experiments). The Notch 1 sorted cells and the remaining cells were cultured and assayed 1 week later for the presence of neurospheres. In the experiments where the cells had been sorted with the Notch 1 antiserum, but not in experiments where the Notch 1 antiserum had been omitted, a higher proportion of spheres formed in the sorted fraction (Figure 3D). The number of spheres formed per cell was 0.6% ± 0.2% (1,172 ± 350 spheres/213,100 ± 25,380 cells) in the input cell population, 0.8% ± 0.2% (553 ± 328 spheres/70,875 ± 39,700 cells) in the wash fraction of the sorting experiments, and 2.8% ± 1.4% (104 ± 41.5 spheres/3,770 ± 1,800 cells) in the sort fraction.

Since only 3% of the cells in the sort fraction formed neurospheres and 95% were Notch 1 positive, it remained a formal possibility that the stem cell population was found within the Notch 1–negative population. To test this possibility, we picked cells in the sort fraction that had no magnetic beads attached (Notch 1–negative cells) and cultured them separately ($n = 70$). Whereas the remaining bead-bound (Notch 1–positive) cells generated neurospheres at the regular frequency, no neurospheres were formed in the cultures of Notch 1–negative cells, demonstrating that the stem cells are found within the Notch 1–positive population. In keeping with this, magnetic beads were seen in the vast majority of neurospheres in the sorting experiments (data not shown).

To ascertain that the immunosorting specifically retained the ependymal cells, we labeled these cells in two independent experiments by intraventricular injection of DiI before sorting. Results from these experiments demonstrated that more than 95% of the cells in the sorted fraction were labeled, whereas only 17% were labeled in the remaining cell fraction, indicating that we indeed were studying the same cell population in both experimental paradigms and that ependymal cells are neural stem cells.

Ependymal Cells Have a Slow Proliferation Rate and Generate a Transit-Amplifying Progenitor Population

The failure to detect incorporation of labeled nucleotides in ependymal cell nuclei after a single or a few injections has previously been interpreted to indicate that these cells do not proliferate in adult mammals (Doetsch et al., 1997). Given the assumption that stem cells proliferate slowly or rarely (Morrison et al., 1997), it is, however, likely that a slow proliferation rate of ependymal cells would go unnoticed if analyzed by these methods.

In order to reexamine the proliferation of ependymal cells, we supplied BrdU continuously through the drinking water over a 2 to 6 week period before analysis. While many ependymal cells lining the lateral wall of the lateral ventricle were labeled (Figures 4A and 4B), ependymal cells lining the roof and the medial wall of the lateral ventricles were less often labeled. Large numbers of BrdU-labeled cells were present in the subventricular zone (Figures 4A and 4B). These cells were often grouped in tight cell clusters, resembling the distribution expected of a clone of cells (Figure 4B). Strikingly, in many cases such a cell cluster was located in close proximity to a labeled ependymal cell (Figure 4B).

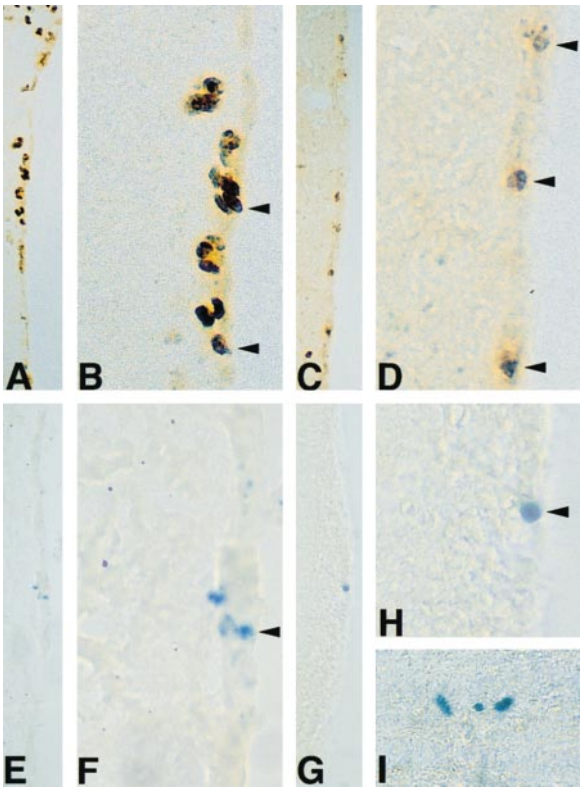

Figure 4. Proliferating Ependymal Cells Generate Subventricular Zone Cells and Olfactory Bulb Cells

Immunohistochemical detection of BrdU in the lateral wall of the lateral ventricle after 2 weeks of continuous BrdU administration (A and B) or 2 weeks administration followed by 1 week without BrdU (C and D). X-gal detection of BAG retrovirus–infected cells 4 days (E and F) or 3 weeks (G–I) after an intraventricular injection. (G) and (H) show the only cell that was labeled in the wall of the lateral ventricle in one animal, and (I) shows labeled cells in the olfactory bulb of the same animal. (B), (D), (F), and (H) show details from (A), (C), (E), and (G), respectively. Arrowheads indicate labeled ependymal cells.

When labeled nucleotides are administered over extended time periods, both rapidly and slowly proliferating cells will be labeled. By allowing the animals to survive for a period of time after the administration of BrdU was completed, rapidly proliferating cells will be given time to dilute the label by continued divisions or to migrate away from the area where they were generated. Therefore, only slowly proliferating stationary cells will retain the label with time. We analyzed animals that had received BrdU continuously over a 2 to 6 week period followed by 1 or 2 weeks without BrdU. In these animals, very few labeled cells were seen in the subventricular zone, indicating that the vast majority of cells had diluted the label by repeated divisions or migrated away (Figures 4C and 4D). In contrast, a substantial number of ependymal cells were still labeled (Figures 4C and 4D). These pulse chase experiments support the notion that ependymal cells are slowly dividing stem cells that generate a transit amplifying population in the subventricular zone.

To characterize the relationship between dividing ependymal cells and subventricular zone cells in greater detail, we used a retrovirus carrying the *lacZ* reporter gene to genetically mark the cells (Sanes, 1989). A very small number of ependymal cells expressed *lacZ* after an intraventricular injection of BAG retrovirus, in line with the rare proliferation of ependymal cells. Serial sections of the brains of injected animals ($n = 13$) demonstrated that 1.9 ± 0.6 (mean $\pm$ SEM) ependymal cells were labeled per brain, a number sufficiently low to allow for clonal analysis of labeled cells. Four days after an injection, the labeled ependymal cells were often associated with a single or a group of *lacZ*-expressing cells in the subventricular zone, suggesting a clonal relationship between these cells (Figures 4E and 4F). Three weeks after the injection, *lacZ* expression was confined to solitary ependymal cells without adjacent subventricular zone cells (Figures 4G and 4H). This suggests that the subventricular zone cells had migrated away, and indeed *lacZ*-expressing cells were seen in the olfactory bulb (Figure 4I). Notably, in two of the animals that survived 3 weeks after the retroviral injection, a single cell was labeled in the wall of the lateral ventricle. In both these animals there were *lacZ*-expressing cells in the olfactory bulb (one of these animals is shown in Figures 4G–4I), in line with an ependymal cell undergoing asymmetric cell division to generate olfactory bulb cells. Collectively, these data support that ependymal cells are neural stem cells and that they are the cellular origin of the transit amplifying population of progenitor cells in the subventricular zone that generate olfactory bulb neurons.

Ependymal Cell Proliferation Increases in Response to Injury

We next studied the distribution of proliferating cells in the spinal cord because the ventricular wall lining the central canal of the spinal cord differs from that in the brain in that it contains no defined subventricular zone. While extremely few cells were labeled after a few injections of BrdU, a substantial number of ependymal cells lining the central canal were labeled after prolonged administration of BrdU (Figures 5A–5C). In contrast to the brain, few labeled cells were seen immediately outside the spinal cord central canal ependyma, corresponding to the lack of a subventricular zone. However, the few labeled cells that were seen close to the central canal often resided in close proximity with a labeled ependymal cell, suggesting that this cell may derive from the ependyma (Figure 5C).

Quantification of the proportion of ependymal cells that proliferate at different time points after an incision in the spinal cord dorsal funiculus revealed an almost 50-fold increase 1 day after the injury compared to uninjured animals (Figures 5D–5G). After the first day, the proliferation gradually declined and was close to normal within 1 month (Figure 5G). The response of ependymal cells to injury was also analyzed by electron microscopy. Interestingly, in two cases, ependymal cells undergoing mitotic cleavage were observed. In both these cells, the cleavage plane was parallel to the luminal surface of the cell (one of these cells is shown in Figure 5H), indicative of asymmetric cell division. To further characterize the orientation of the mitotic cleavage plane of ependymal

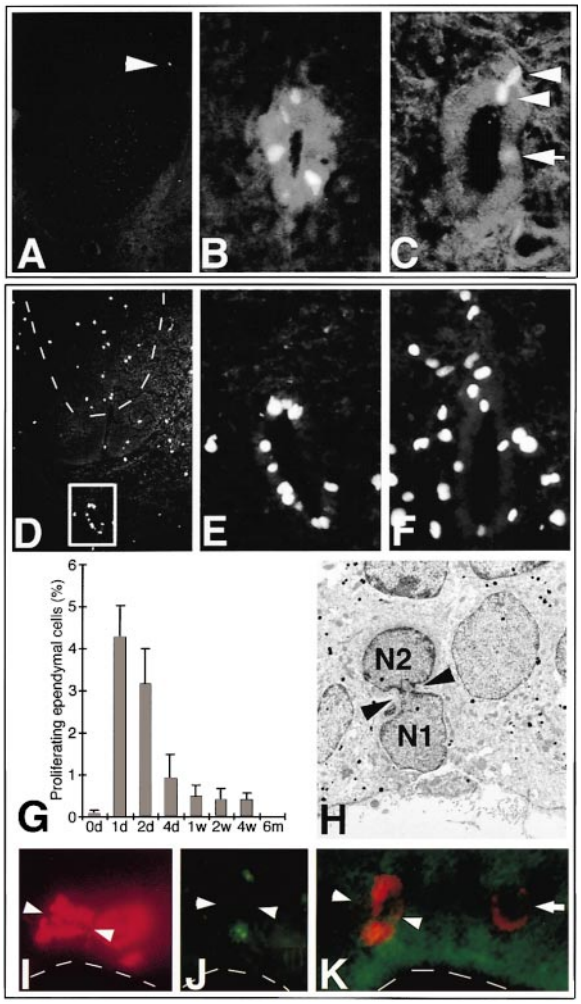

Figure 5. Proliferation Kinetics and Orientation of the Mitotic Cleavage Plane after Spinal Cord Injury

Proliferation of ependymal cells in the uninjured (A–C) and injured (D–K) spinal cord. Labeled cells are very rarely encountered in the uninjured spinal cord when BrdU is administered over 8 hr before sacrifice (A). A single cell is indicated with an arrowhead in (A). In contrast, when BrdU is given continously over 2 weeks, ependymal cells are often labeled (B and C). In some sections, a labeled ependymal and subependymal cell reside in very close proximity (arrowheads in [C]). An additional labeled ependymal cell that is not in the plane of focus is indicated with an arrow in (C). Many ependymal cells are labeled 1 (D and E) and 2 (F) days after an incision in the spinal cord dorsal funiculus when BrdU is given over the last 8 hr before sacrifice. (E) The central canal from the animal in (D) at higher magnification. (G) The proliferation of ependymal cells in response to injury was quantified by calculating the proportion of spinal cord ependymal cells that incorporate BrdU administered during the last 8 hr before sacrifice (mean from 3–5 rats at each time point; error bars show SEM). (H) Immunoelectron microscopic localization of nestin (black gold-silver complexes) in a dividing ependymal cell. Two distinct nuclei (N1 and N2) are seen. In (I–K), the orientation of the mitotic cleavage plane is visualized by immunohistochemical localization of the mitosis-specific phosphorylated form of histone H3 (I and K) or the spindle pole body protein pericentrin (J) 30 hr after injury. Notch 1 immunoreactivity detected with the antibody against the intracellular domain of Notch 1 is shown in green in (K). One cell in (I) and one in (K) show chromosomal condensation and histone H3 immunoreactivity, but chromosomal segregation has not yet been initiated (arrow in [K]). Arrowheads in (H–K) indicate the mitotic cleavage plane, and the broken lines in (I–K) outline the luminal surface of the ependymal cells.

cells, we analyzed sections from animals that had a spinal cord injury 30 hr earlier (330 sections from nine animals). The orientation of the mitotic spindle was determined by immunohistochemical localization of the spindle pole body protein pericentrin or the mitosis-specific phosphorylated form of histone H3. Of the cells with an identifiable cleavage plane, 83% (24/29) had a cleavage plane parallel and 17% (5/29) perpendicular to the luminal surface of the cell (Figures 5I and 5J), suggesting that the majority of ependymal cells undergo asymmetric cell division. We also found that Notch 1 immunoreactivity, as in the brain ependymal cells of uninjured animals (Figure 3), was highly enriched in a crescent in the luminal membrane of ependymal cells in the injured spinal cord (Figure 5K), suggesting that Notch 1 is inherited predominantly by the daughter cell closest to the lumen after asymmetric cell division. As Notch activity has been shown to maintain cells in a nondifferentiated state (Artavanis-Tsakonas et al., 1995), the retention of Notch 1 in the luminal daughter cell may play a role in keeping this cell as a stem cell. Taken together, these data clearly show that ependymal cells of the spinal cord are proliferative, that they respond to injury by increasing their proliferation rate, and that the majority of ependymal cells undergo asymmetric cell division.

Generation of Astrocytes from Ependymal Cells after Injury

To study the fate of the progeny generated from ependymal cells in response to a lesion, we made lesions in animals that had received a DiI injection labeling the ependymal cells prior to the lesion (schematically depicted in Figure 1B). After a dorsal funiculus incision, an increasing number of DiI-labeled cells was seen progressively further out from the ependymal layer over the first 4 weeks after the injury, mimicking the nestin expression pattern (Figure 6). DiI-labeled cells were abundant in the forming scar tissue within 1 week after the lesion and persisted there for at least 1 year (data not shown). When the incision was instead made in the lateral funiculus, the DiI-labeled cells did not migrate dorsally but laterally toward the injury (data not shown).

Within the scar tissue forming at the injury, the vast majority of the DiI-labeled cells showed immunoreactivity to glial fibrillary acidic protein (GFAP), indicating that most of the progeny from ependymal cells had differentiated to astrocytes (Figures 6G and 6H). The DiI-labeled astrocytes were intermingled with non-DiI-labeled astrocytes, which thus presumably had not been generated from ependymal cells at the time of the injury. No DiI-labeled cells expressed the neuronal or oligodendrocyte markers βIII-tubulin or O4, respectively (data not shown). To exclude the possibility that the DiI-labeled cells in the injured spinal cord had been labeled through the subarachnoid space that is penetrated at the injury, cerebrospinal fluid was aspirated from the subarachnoid space 10 days after an intraventricular injection and the fluid was analyzed for the presence of free DiI by adding it to astrocyte cultures. No labeling of the cells could be detected (data not shown). Furthermore, in control animals that received a DiI injection in the subarachnoid

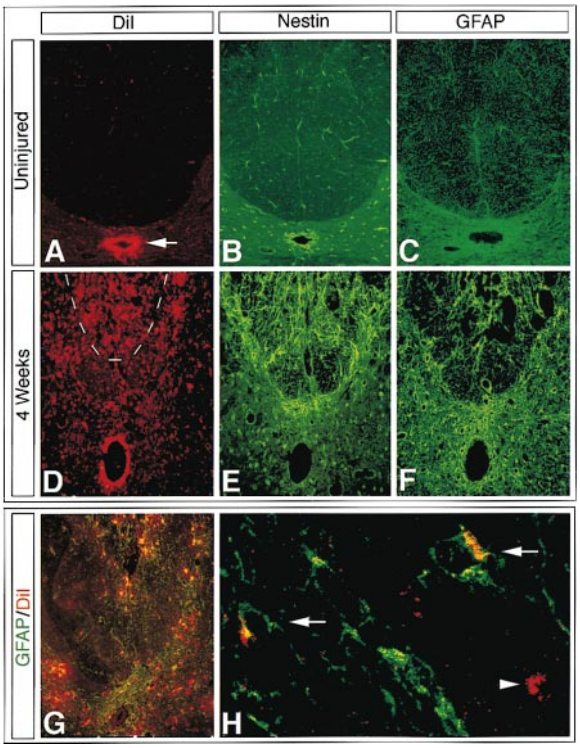

Figure 6. Generation of Astrocytes from Ependymal Cells after Spinal Cord Injury

Adult rats received intraventricular injections of DiI, and the distribution of DiI, nestin, and GFAP immunoreactivity was analyzed in the uninjured (A–C) and injured (D–H) spinal cord. The animals shown in (D–F) and (G and H) were subjected to an incision in the dorsal funiculus 4 or 2 weeks prior to analysis, respectively. DiI and nestin immunoreactivity are shown in the same sections and GFAP immunoreactivity in an adjacent section in (A–F). The approximate delineation of the injured area is indicated by the broken line in (D). (G) shows DiI (red) and GFAP immunoreactivity (green) in the dorsal funiculus. (H) Confocal laser scanning microscope visualization of DiI and GFAP immunoreactivity in the scar tissue. Two GFAP-immunoreactive DiI-labeled cells are indicated by arrowheads, and a DiI-labeled cell that does not show any detectable GFAP is indicated with an arrow.

space 10 days prior to a dorsal funiculus incision, resulting in strong labeling of the meninges, labeled cells were restricted to the surface of the scar tissue (data not shown). These experiments demonstrate that ependymal cells generate astrocytes in response to injury and that these cells participate in scar formation.

Discussion

New neurons are continuously generated in specific regions of the adult mammalian brain. These neurons are derived from multipotent stem cells, the identity of which has been enigmatic. We show here that ependymal cells are neural stem cells and that they generate olfactory bulb neurons. Ependymal cells divide rarely, but after an injury the proliferation rate increases and their progeny migrates to the site of the lesion, where they differentiate to astrocytes.

Are All Ependymal Cells Stem Cells?

The present data demonstrate that a neural stem cell is localized in the ependymal layer, but are all ependymal cells stem cells? Several studies have examined the morphology of the ependymal layer at the ultrastructural level and concluded that it is composed of a morphologically homogeneous cell population (reviewed by Del Bigio, 1995). In agreement with this, the stem cells that were identified in the ependymal layer by BrdU retention did not show any obvious features distinguishing them from other cells in the ependymal layer. Furthermore, the expression of *nestin* and *Notch 1*, genes that are expressed in embryonic neural stem cells and that may serve as markers for immature neural cells, appears to be uniform within the ependymal layer. It should, however, be noted that the long-term BrdU-labeling experiments demonstrate that only scattered ependymal cells have divided over a period of several weeks. This can be interpreted in two ways: either only a subpopulation of ependymal cells is endowed with stem cell properties or, alternatively, many or all have stem cell properties but the majority are quiescent. Interestingly, in the cell-sorting experiments, 3% of the cells in the Notch 1 sorted fraction generated neurospheres, and 6% of the ependymal cells cultured as single cells formed neurospheres. These numbers are similar to the proportion (7%) of embryonic ventricular zone cells that have stem cell properties in vitro (Davis and Temple, 1994). The long-term BrdU-labeling studies and the retroviral lineage experiments may argue against only a small subpopulation of ependymal cells being stem cells. In the experiments where animals received BrdU over a long time period followed by a 1 week chase period, as well as in the animals that were sacrificed 3 weeks after a retroviral injection, no clusters of labeled cells were seen in the subventricular zone. This indicates that the stem cells that had been labeled by BrdU incorporation or retroviral infection did not divide again, since in that case their progeny would have inherited the label and clusters of labeled transit amplifying progenitors would have been seen. This experiment may suggest that different groups of stem cells are active at different time points, a hypothesis that is in accordance with data from the hematopoetic system where many stem cells are quiescent at a given time, but successive subsets of stem cells are activated in the animal over time (Lemischka et al., 1986).

Are All Stem Cells Ependymal Cells?

In addition to stem cells associated with the ventricular system, there is evidence for the existence of neural stem cells also in the hippocampus of adult mammals (Gage et al., 1995). The identity and exact localization of hippocampal stem cells has not been precisely determined, and it is thus not clear whether they represent a separate population or if they also derive from the ependyma. The dentate gyrus of the hippocampus, where the most prominent neurogenesis is seen, is located in proximity to part of the lateral ventricle. Moreover, adjacent to the hippocampus, scattered groups of ependymal cells are found. These cells are left behind when the lateral ventricles, which extend over the hippocampus during development, later retract (Kawamata et al., 1995).

Our data, however, leaves open the possibility that there are other independent populations of stem cells in addition to ependymal cells in the adult central nervous system. Indeed, not all (89%) stem cell spheres that formed in culture from tissue derived from animals in which ependymal cells had been labeled by a DiI injection were labeled. While this may be a result of very weak labeling of some cells that have a small cell surface area facing the ventricular lumen, it could also indicate that cells outside the ependymal layer can generate neurospheres, and we cannot rule out the existence of an additional independent stem cell population in the subventricular zone.

Symmetric versus Asymmetric Cell Division

During embryonic brain development, the orientation of the mitotic cleavage plane in a ventricular zone cell plays a role in regulating the rate of neurogenesis. A cleavage plane perpendicular to the luminal surface of the cell generates two daughter cells that will stay in the ventricular zone (Chenn and McConnell, 1995). If the cleavage plane instead is parallel to the luminal surface of the cell, one cell will remain in the ventricular zone, while the other will migrate and undergo neuronal differentiation (Chenn and McConnell, 1995).

Three observations provide information regarding the plane of ependymal cell division. First, the identification of rarely dividing ependymal cells by long-term BrdU administration revealed that the absolute majority of these cells did not have a labeled neighboring ependymal cell. If the ependymal cells had undergone cell divisions within the plane of the ependyma to generate two ependymal stem cells, one would expect to frequently find pairs of labeled ependymal cells. In contrast, one or a group of BrdU-labeled cells were often seen in the subventricular zone just adjacent to a labeled ependymal cell. Second, retroviral labeling did not reveal any clones containing two or more ependymal cells, which would have been expected if the infected cell underwent symmetric cell division. Third, when ependymal cell proliferation is increased in response to injury, the majority of the cells (83%) have a cleavage plane parallel to the luminal surface of the ependymal cell, as expected for asymmetric cell division. Based on these data, we propose a model in which an ependymal cell can undergo asymmetric cell division to generate one daughter cell that stays as an undifferentiated stem cell in the ependymal layer and one cell that moves into the subventricular zone (Figure 7). In the wall of the lateral ventricle, the daughter cell in the subventricular zone will then undergo several rounds of rapid proliferation to generate a larger number of neuronal progenitor cells that migrate to the olfactory bulb (Figure 7).

Participation of Neural Stem Cells in Injury Reactions

It is interesting to observe that only astrocytes and not neurons are formed from stem cells in response to injury. The molecular environment changes radically after a central nervous system injury; in addition to altered gene expression in cells residing close to the injury, the breakdown of the blood–brain barrier with the concomitant influx of macrophages at the site of a lesion results in the presence of many growth factors and cytokines that may affect stem cell proliferation and differentiation. For example, basic fibroblast growth factor (bFGF), which is a potent mitogen for neural stem cells (Gage et al., 1995), is expressed in the injured spinal cord (Finkelstein et al., 1988). Moreover, expression of ciliary neurotrophic factor (CNTF), which induces astrocytic differentiation of neural stem cells in vitro (Johe et al., 1996), is likewise triggered in response to injury (Ip et al., 1993) and may impel astrocytic differentiation in vivo in response to injury. Future elucidation of the molecular mechanisms directing the proliferation and differentiation of stem cells in response to injury may enable the development of strategies to reduce scar formation or to stimulate neurogenesis.

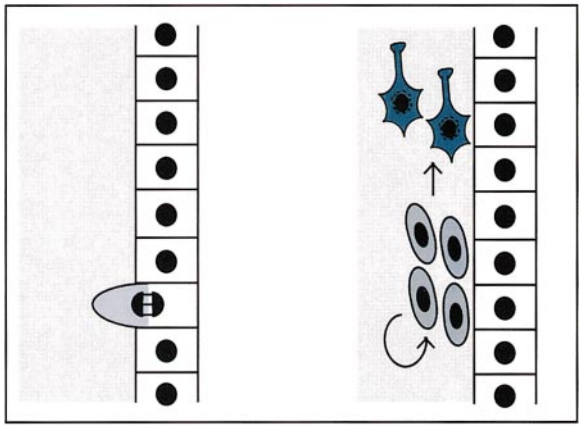

Figure 7. Model for the Generation of Differentiated Progeny from an Ependymal Stem Cell

In this model, an ependymal cell undergoes asymmetric cell division to generate a subventricular zone progenitor cell (light blue). The progenitor cell undergoes several rounds of division to generate migratory neuronal precursor cells (dark blue).

Cell loss is a common factor in many types of nervous system disorders. Transplantation of cells from human embryos or animals has been tested with some encouraging results (Gage, 1998). However, these methods have several problems, both of ethical and immunological nature. The utilization of endogenous stem cells to generate new neurons or glial cells in the treatment of nervous system diseases is therefore a tantalizing possibility (McKay, 1997). A recent study has shown that stem cells from the embryonic and adult nervous systems may be very similar and that their differentiation is regulated indistinguishably by a number of growth factors and cytokines (Johe et al., 1996). Much of the rapidly emerging data from studies on the generation of distinct cell types in the developing nervous system may thus be applicable to adult neural stem cells and may open new perspectives to steer the differentiation to various cell fates in the adult central nervous system.

Experimental Procedures

Labeling and Viral Infection of Ependymal Cells

Unilateral stereotaxic injections of 10 μl (rats) or 3 μl (mice) of 0.2% w/v DiI [1,1′-dioctadecyl-6,6′-di(4-sulfophenyl)-3,3,3′,3′-tetramethylindocarbocyanine, Molecular Probes] in DMSO, 10^9 CMV-*lacZ* adenovirus plaque-forming units in 10 mM Tris-HCl, or 10^4 BAG retroviral particles (Price et al., 1987) in 10 mg/ml Polybrene were made

slowly 0.9 mm (rats) or 0.5 mm (mice) posterior and 1.4 mm (rats) or 0.7 mm (mice) lateral to Bregma and 3.6 mm (rats) or 2 mm (mice) below the dura mater into the lateral ventricle.

Cell Cultures

The lateral wall of the lateral ventricles and the spinal cord were enzymatically dissociated in 0.7 mg/ml hyaluronic acid, 0.2 mg/ml kynurenic acid, and 1.33 mg/ml trypsin in HBSS with 2 mM glucose at 37°C for 30 min. The cells were centrifuged at 200 g for 5 min, resuspended in 0.9 M sucrose in 0.5× HBSS, and centrifuged for 10 min at 750 g. The cell pellet was resuspended in 2 ml of culture medium, placed on top of 10 ml 4% BSA in EBSS solution, and centrifuged at 200 g for 7 min, followed by washing in DMEM/F12. The culture medium consisted of 20 ng/ml EGF, 20 ng/ml bFGF, B27 supplement, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in DMEM-F12 medium. Single cells were cultured in 96-well dishes in 50% neurosphere-conditioned medium and 50% fresh medium.

Cell Sorting

The dissociation solution consisted of 0.075% collagenase type 1, 0.075% hyaluronidase, and 500 U/ml DNaseI in 0.2 M PIPES. The cells were resuspended in 1:100 anti-Notch 1 antiserum (Mitsiadis et al., 1995) and incubated for 20 min at 4°C. After rinsing in a large volume of DMEM/F12, the cells were resuspended in 100 μl of culture medium containing magnetic bead–conjugated goat anti-rabbit antiserum (1.8–2.1 × 10^7 beads/100 μl, Dynal) and were incubated for 20 min at 4°C. Subsequently, 2 ml of culture medium was added to the tube that was placed in the magnetic separator. After 2 min, the supernatant containing cells that had not bound magnetic beads was collected and 2 ml culture medium was added to the tube. The magnet was then removed and the cells that had bound magnetic beads were collected.

Immunohistochemistry

Cryostat sections and cultured cells were incubated with primary antibodies 1 hr at 37°C or overnight at 4°C, rinsed in PBS, and incubated with secondary antiserum for 45 min at room temperature. The following primary antibodies were used: rabbit anti-CXADR (1:50, provided by L. Philipson), rabbit anti-GFAP (1:250, Dako), rabbit anti-nestin (1:2000, Dahlstrand et al., 1992), mouse monoclonal anti-Notch 1 intracellular domain (1:20, bTAN20, Zagouras et al., 1995), rabbit anti-Notch 1 extracellular domain (1:100, Mitsiadis et al., 1995), mouse monoclonal anti-βIII-tubulin (1:500, BAbCO), mouse monoclonal anti-O4 (1:5–10, Boehringer Mannheim), rabbit anti-pericentrin (1:300, BAbCO), and rabbit antiphosphorylated histone H3 (1:1000, Upstate Biotechnology). Electron microscopic localization of nestin was done as in Frisén et al. (1995).

Proliferation Studies

BrdU was administered through the drinking water (1 mg/ml) or by intraperitoneal injection (150 mg/kg) and detected with mouse anti-BrdU (1:200, Dako).

Spinal Cord Injuries

A T2 laminectomy was performed, and the dorsal funiculus was cut transversely as described (Frisén et al., 1995).

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References

Altman, J., and Das, G.D. (1965). Autoradiographic and histological evidence of postnatal neurogenesis in rats. J. Comp. Neurol. *124*, 319–335.

Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M.E. (1995). Notch signaling. Science *268*, 225–232.

Chenn, A., and McConnell, S.K. (1995). Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogensis. Cell *82*, 631–641.

Dahlstrand, J., Collins, V.P., and Lendahl, U. (1992). Expression of the class VI intermediate filament nestin in human central nervous system tumors. Cancer Res. *52*, 5334–5341.

Davidson, B.L., and Bohn, M.C. (1997). Recombinant adenovirus: a gene transfer vector for study and treatment of CNS disease. Exp. Neurol. *144*, 125–130.

Davis, A.A., and Temple, A. (1994). A self-renewing multipotential stem cell in embryonic rat cerebral cortex. Nature *372*, 263–266.

Del Bigio, M.R. (1995). The ependyma: a protective barrier between brain and cerebrospinal fluid. Glia *14*, 1–13.

Doetsch, F., Garcia-Verdugo, J.M., and Alvarez-Buylla, A. (1997). Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J. Neurosci. *17*, 5046–5061.

Finkelstein, S.P., Apostolides, P.J., Caday, C.G., Prosser, J., Philips, M.F., and Klagsburn, M. (1988). Increased basic fibroblast growth factor (bFGF) immunoreactivity at the site of focal brain wounds. Brain Res. *460*, 253–259.

Frisén, J., Johansson, C.B., Török, C., Risling, M., and Lendahl, U. (1995). Rapid, widespread, and longlasting induction of nestin contributes to the generation of glial scar tissue after CNS injury. J. Cell Biol. *131*, 453–464.

Gage, F.H. (1998). Cell therapy. Nature *392* suppl., 18–24.

Gage, F.H., Ray, J., and Fisher, L.J. (1995). Isolation, characterization, and use of stem cells from the CNS. Annu. Rev. Neurosci. *18*, 159–192.

Goldman, S.A., and Luskin, M.B. (1998). Strategies utilized by migrating neurons of the postnatal vertebrate forebrain. Trends Neurosci. *21*, 107–114.

Gritti, A., Parati, E.A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., Faravelli, L., Morasutti, D.J., Roisen, F., Nickel, D.D., and Vescovi, A.L. (1996). Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J. Neurosci. *16*, 1091–1100.

Ip, N.Y., Wiegand, S.J., Morse, J., and Rudge, J.S. (1993). Injury-induced regulation of ciliary neurotrophic factor mRNA in the adult brain. Eur. J. Neurosci. *5*, 25–33.

Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., and McKay, R.D.G. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. *10*, 3129–3140.

Kawamata, S., Stumpf, W.E., and Bidmon, H.J. (1995). Adhesion and fusion of ependyma in rat brain. Acta Anat. *152*, 205–214.

Lemischka, I.R., Raulet, D.H., and Mulligan, R.C. (1986). Developmental potential and dynamic behavior of hemapoetic stem cells. Cell *45*, 917–927.

Lendahl, U., Zimmerman, L., and McKay, R. (1990). CNS stem cells express a new class of intermediate filament protein. Cell *60*, 585–595.

Lois, C., and Alvarez-Buylla, A. (1993). Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc. Natl. Acad. Sci. USA *90*, 2074–2077.

Lois, C., and Alvarez-Buylla, A. (1994). Long-distance neuronal migration in the adult mammalian brain. Science *264*, 1145–1148.

Luskin, M.B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron *11*, 173–189.

McKay, R. (1997). Stem cells in the central nervous system. Science *276*, 6671.

Mitsiadis, T.A., Lardelli, M., Lendahl, U., and Thesleff, I. (1995). Expression of Notch1, 2 and 3 is regulated by epithelial-mesenchymal interactions and retinoic acid in the developing mouse tooth and associated with determination of ameloblast cell fate. J. Cell Biol. *130*, 407–418.

Morrison, S.J., Shah, N.M., and Anderson, D.J. (1997). Regulatory mechanisms in stem cell biology. Cell *88*, 287–298.

Morshead, C.M., Reynolds, B.A., Craig, C.G., McBurney, M.W., Staines, W.A., Morassutti, D., Weiss, S., and van der Kooy, D. (1994). Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron *13*, 1071–1082.

Palmer, T.D., Takahashi, J., and Gage, F.H. (1997). The adult rat hippocampus contains primordial neural stem cells. Mol. Cell. Neurosci. *8*, 389–404.

Price, J., Turner, D., and Cepko, C. (1987). Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc. Natl. Acad. Sci. USA *84*, 156–160.

Reynolds, B.A., and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian nervous system. Science *255*, 1707–1710.

Reynolds, B.A., and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic precursor is a stem cell. Dev. Biol. *175*, 1–13.

Sanes, J.R. (1989). Analysing cell lineage with a recombinant retrovirus. Trends Neurosci. *12*, 21–28.

Svendsen, C.N., Fawcett, J.W., Bentlage, C., and Dunnett, S.B. (1995). Increased survival of rat EGF-generated precursor cells using B27 supplemented medium. Exp. Brain Res. *102*, 407–414.

Tomko, R.P., Xu, R., and Philipson, L. (1997). HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc. Natl. Acad. Sci. USA *94*, 3352–3356.

Weinmaster, G., Roberts, V.J., and Lemke, G. (1992). Notch2: a second mammalian Notch gene. Development *116*, 931–941.

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A.C., and Reynolds, B.R. (1996). Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. J. Neurosci. *16*, 7599–7609.

Zagouras, P., Stifani, S., Blaumueller, C.M., Carcangui, M.L., and Artavanis-Tsakonas, S. (1995). Alterations in Notch signaling in neoplastic lesions of the human cervix. Proc. Natl. Acad. Sci. USA *92*, 6414–6418.