In vivo clonal analyses reveal the properties of endogenous neural stem cell proliferation in the adult mammalian forebrain

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SUMMARY

The adult mammalian forebrain contains a population of multipotential neural stem cells in the subependyma of the lateral ventricles whose progeny are the constitutively proliferating cells, which divide actively throughout life. The adult mammalian brain is ideal for examining the kinetics of the stem cells due to their strict spatial localization and the limited and discrete type of progeny generated (constitutively proliferating cells). Clonal lineage analyses 6 days after retrovirus infection revealed that under baseline conditions 60% of the constitutively proliferating cells undergo cell death, 25% migrate to the olfactory bulb and 15% remain confined to the lateral ventricle subependyma (where they reside for approximately 15 days). Analysis of single cell clones 31 days after retroviral infection revealed that the stem cell divides asymmetrically to self-renew and give rise to constitutively proliferating cells. Following repopulation of the depleted subependyma the average clone size is 2.8 times larger than control, yet the absolute number of cells migrating to the olfactory bulb is maintained and the stem cell retains its asymmetric mode of division. The number of neural stem cells in the adult forebrain 33 days after repopulation of the subependyma was estimated using bromodeoxyuridine labeling of subepenydmal cells. There were calculated to be 1200-1300 cells between the rostral corpus callosum and rostral anterior commissure; these data support a lineage model similar to those based on stem cell behavior in other tissue types.

Key words: Neural stem cell, Mammalian forebrain, Clonal analysis, Mouse

INTRODUCTION

The adult mammalian brain contains both neural stem cells and their progeny the constitutively proliferating cells, both of which line the lateral ventricles in the forebrain (Smart, 1961; Morshead and van der Kooy, 1992; Morshead et al., 1994). Compared to both the embryonic brain and to other tissue types, the adult brain is an ideal system in which to study the kinetics of stem cells because the stem cells and their progeny are precisely spatially localized within the ventricular lining and there are relatively few progeny, all of which show limited differentiation under baseline conditions in the adult.

The subependyma, which lines the lateral ventricles and extends rostrally towards the olfactory bulbs (Smart, 1961; Sturrock and Smart, 1980; Morshead and van der Kooy, 1992), contains the mitotically active progeny (the constitutively proliferating cells) of the relatively quiescent stem cells. The subependyma is derived from the subventricular zone, which gives rise during embryonic and early postnatal development to forebrain neurons and glia (Smart, 1961). In the adult this region shrinks to become a few cell layers thick and contains a heterogeneous population of cells. The constitutively proliferating cells constitute approximately 10% of all the

subependymal cells and have a cell-cycle time of approximately 12.7 hours (Morshead and van der Kooy, 1992). The constitutively proliferating population proliferates in a steady state mode of division whereby one of the progeny continues to divide and the other either undergoes cell death (Morshead and van der Kooy, 1992) or migrates to the olfactory bulb where it differentiates into a neuron (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Lois et al., 1996). The relative frequency of cell death versus survival and differentiation into olfactory bulb neurons is unknown.

The adult subependyma also contains a population of neural stem cells (Morshead et al., 1994). In vivo when the constitutively proliferating cells have been depleted by using high doses of tritiated thymidine ([3H]thy) to kill the mitotically active cells, a subpopulation of subependymal cells is able to completely replenish the constitutively proliferating population over 8 days (Morshead et al., 1994). The subependymal cells capable of this repopulation have been isolated in vitro in the presence of epidermal growth factor (EGF) (Morshead et al., 1994; Reynolds and Weiss, 1996) and they display the cardinal properties of stem cells: proliferation, self-renewal and the production of differentiated progeny (astrocytes, oligodendrocytes and neurons). Hence, the

subependyma contains a population of neural stem cells that are normally relatively quiescent and whose progeny are the constitutively proliferating cells.

Probably the best understood stem cell in the adult body is that in the haemopoietic system. The haemopoietic stem cell generates the terminally differentiated cells of the blood (Iscove, 1990) through a series of proliferating progenitor cells known as transit amplifying cells. Besides replacing itself, asymmetric divisions of the haemopoietic stem cell generate primitive progenitors that differentiate into mature blood cells (Ogawa et al., 1983; Dexter and Spooncer, 1987). We have undertaken clonal analyses of the lineage relationships among the cells within the subependyma to discern if neural stem cells utilize the same lineage relationships as haemopoietic stem cells under baseline conditions and during repopulation in the adult. Presently there are no selective markers for the different cells types such as stem cells, transit amplifying cells and constitutively proliferating cells within the subependyma of the adult brain. Accordingly, to understand the lineage relationship between these cell types we undertook a clonal analysis and examined the size and distribution of the cells within individual clones. By injecting limiting dilutions of a replication-deficient retrovirus (containing a β-galactosidase-producing marker gene) such that only one cell per hemisphere is infected, we have addressed some of the fundamental features of stem cell kinetics and the fates of their progeny, the constitutively proliferating cells. We have found that approximately 60% of the progeny of the constitutively proliferating cells undergo cell death, 25% migrate to the olfactory bulbs and 15% remain confined to the lateral ventricle subependyma. The average duration of residence of a constitutively proliferating cell within the subependyma lining the lateral ventricle is 15 days. Our data reveal that stem cells divide asymmetrically to self renew and give rise to exponentially dividing transit amplifying cells during repopulation of the constitutively proliferating subependymal cells, similar to haemopoietic stem cells. We have labeled the proliferating stem cells during repopulation and estimated that there are approximately 1200 stem cells in the adult mouse forebrain (within the area bounded anteriorly by the rostral tip of the crossing of the corpus callosum and posteriorly by the rostral tip of the crossing of the anterior commissure) constituting 0.2-0.4% of the entire subependymal population.

MATERIALS AND METHODS

Retrovirus injections

A replication-deficient retrovirus engineered to introduce a β-galactosidase-encoding marker gene (*E. coli lacZ*) into the genome of an infected host cell was harvested from the supernatant of confluent psi2BAGalpha cells (ATCC #CRL-9560) for 48 hours at 32°C, 0.45 μm filtered, supplemented with 8 μg/ml polybrene, aliquotted and stored at −70°C (Price et al., 1987). The viral concentrate titer was approx. 10^6/ml, active virions are determined by infection of NIH 3T3 cells. Adult male CD1 mice were anaesthetized using sodium pentobarbital (65 mg/kg, i.p.) and bilateral stereotaxic injections of 0.27 μl of retrovirus were made into the lateral ventricles using a 1 μl Hamilton syringe. The coordinates for the injections were AP +4.0 mm anterior to lambda, L +0.9 mm and DV −1.8 mm below the dura with the mouthbar at −2 mm below the interaural line. Unilateral injections of as much as 1.0 μl of retrovirus into the lateral ventricle

do not result in infection of cells in the contralateral hemisphere (unpublished observations), hence each hemisphere was considered a separate sample. Initial experiments were done to determine the volume of retrovirus which resulted in single infections per brain. Animals received 0.9 μl (*n*=6), 0.5 μl (*n*=6) or 0.2 μl (*n*=8) injections of retrovirus into the lateral ventricle and survived for 1 day. The number of clones per brain was determined for each of the volumes, based solely on the distance between cells as a criterion for clonality (Fig. 1). A best fit curve was then used to determine the approximate volume of retrovirus required in vivo to result in single cell infections. All animals received injections of the same batch of retrovirus. Control animals were killed (by perfusion) 36 hours (*n*=33), 6 days (*n*=48) or 31 days (*n*=66) after injection of the retrovirus. Two other groups of animals received a series of intraperitoneal injections of [³H]thy (three injections, one every 4 hours; 0.8 ml per injection; 1.0 mCi/ml; specific activity 45-55 Ci/mmol; ICN Biomedical), 2 days prior to the retrovirus injections, to kill the constitutively proliferating subependymal cells. Previous work (Becker et al., 1965; Lajtha et al., 1969; Morshead et al., 1994) has established that the constitutively proliferating subependymal cells are killed by the high doses of [³H]thy due to intranuclear radiation (indicated by the specific activity) combined with the high dose of [³H]thy. Most importantly, we have shown that at 1 and 2 days post-kill the heavily tritiated-thymidine-labeled cells disappear from the subependyma rather than proliferate and dilute the label (Morshead et al., 1994; 1997). Animals receiving a kill were perfused 6 days (*n*=73) or 31 days (*n*=66) after the retrovirus injections (8 and 33 days post-kill, respectively).

Perfusion

At the end of the experimental period animals were overdosed with anesthetic and transcardially perfused with 2% paraformaldehyde in 0.1 M phosphate buffer. Brains (including the olfactory bulbs) were removed and fixed overnight in 20% sucrose at 4°C.

Histological procedure

Serial sections, 60 μm thick, were cut at on a cryostat (−20°C) and
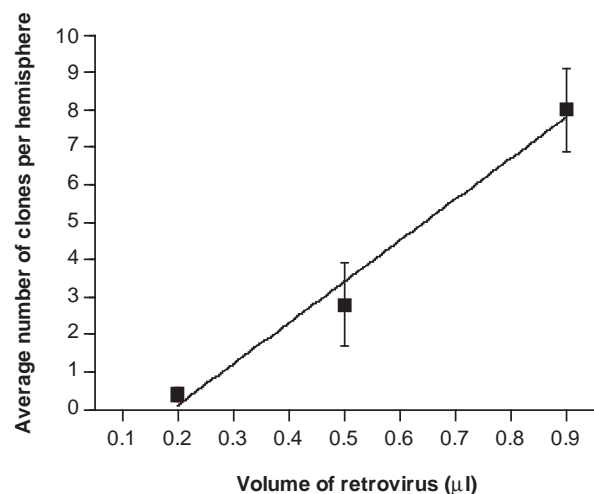
Fig. 1. The linear relationship (R coefficient is 0.98) between the number of clones per brain hemisphere and the volume of retrovirus injected into the lateral ventricles of adult mice. Animals were perfused 24 hours after injections of 0.2 μl (*n*=8), 0.5 μl (*n*=6) or 0.9 μl (*n*=6) of retrovirus. The numbers of clones per hemisphere were counted using the distance between cells as the criterion for establishing clonality (cells within 180 μm of each other were considered part of the same clone). These data were used to estimate the volume of retrovirus which would result in single clones per hemisphere. Data represent means ± s.e.m.

mounted directly onto gelled slides. To visualize the retrovirally infected cells the slides were stained for β-gal at 37°C overnight in the dark in a solution containing X-gal (1 mg/ml, Promega) in 0.1 M phosphate buffer containing $MgCl_2$ (2 mM), sodium deoxycholate (0.01%), NP40 (0.02%), potassium ferrocyanide (50 mM) and potassium ferricyanide (50 mM). Slides were rinsed in 3% dimethylsulfoxide in 0.1 M phosphate buffer.

The number of retrovirally labeled cells was counted throughout the entire hemisphere from the olfactory bulbs rostrally and extending caudally to the rostral tip of the crossing of the anterior commissure. Cells were grouped into two categories based on their location. (1) Lateral ventricle cells which were found within the subependyma surrounding the lateral ventricle (lv) and (2) olfactory bulb cells, which included cells within the olfactory bulb as well as cells along the pathway to the olfactory bulb (ob).

Total number of stem cells

To estimate the total number of EGF-responsive stem cells that reside in the adult brain (Morshead et al., 1994), animals were divided into 3 groups. One group of animals (n=3) received a series of bromodeoxyuridine (BrdU) injections on day 0 (5 injections i.p., 0.2 ml every 2 hours of 18 mg/ml BrdU in 0.1 M phosphate buffer; Sigma) and were perfused 0.5 hours after the last BrdU injection. A second and third group of animals (n=4 each group) received high doses of [^{3}H]thy on day 0 as described above in order to kill the constitutively proliferating cells. This was followed by the same series of BrdU injections on day 2 post-kill (a time when 50% of the stem cells are proliferating (Morshead et al., 1994) and they were allowed to survive for 0.5 hours or 31 days, respectively for the second and third groups. The animals were killed with an anesthetic overdose and transcardially perfused with 4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer, pH 6.9. Brains were postfixed in the perfusing solution for 90 minutes at 4°C and then cryoprotected for at least 24 hours in 10% sucrose in 0.1 M phosphate buffer. Serial 14 μm coronal cryosections were mounted directly onto chromium-aluminum subbed slides. Sections were incubated in 1 M HCl for 30 minutes at 60°C to denature the DNA. Slides were incubated for 48 hours in rat anti-BrdU antibody (1:100; Seralab) followed by fluorescein isothiocyanate-conjugated donkey anti-rat antibody (1:100, Jackson). The total number of BrdU-labeled cells was counted using the optical dissector method (Coggeshall and Lekan, 1996) in every 10th section from the rostral tip of the crossing of the corpus callosum rostrally and extending caudally to the rostral tip of the crossing of the anterior commissure. An average number of cells/section was then calculated and multiplied by the total number of sections extending from rostral to caudal (average number of 14 um sections per brain region = 91) For an estimate of the total number of subependymal cells within this same region, the absolute number of subependyma cells was calculated surrounding the ventricle using Nissl cell counts from previous work (Morshead and van der Kooy, 1992).

RESULTS

Clone size reveals the number of constitutively proliferating progeny which migrate to the olfactory bulb

A replication-deficient retrovirus containing the β-galactosidase gene was injected bilaterally into the lateral ventricles of adult mice in order to label proliferating cells within the subependyma (Fig. 2A). For a clonal analysis it was necessary that the amount of retrovirus injected would result in the infection of a single cell (one of the progeny of the division of the infected cell would express the β-galactosidase
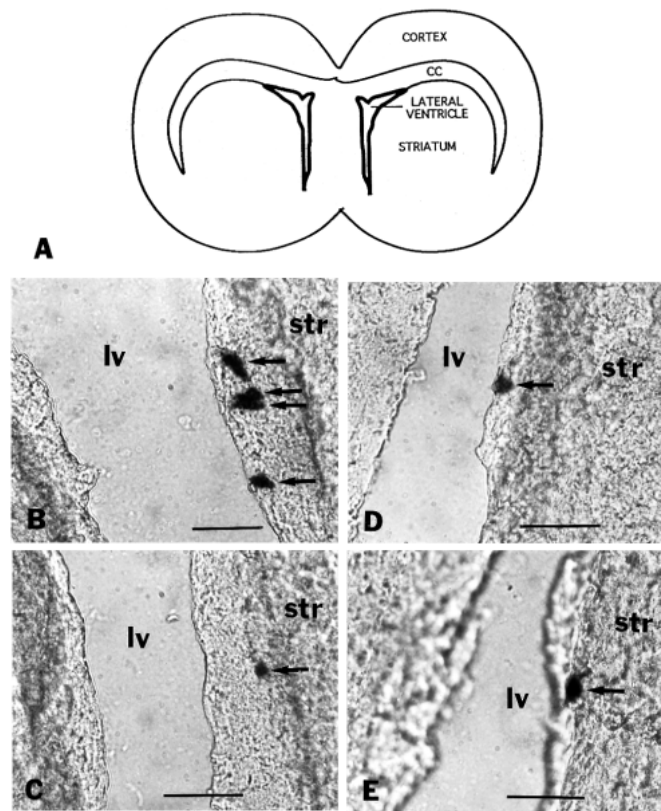
Fig. 2. (A) Schematic representation of a coronal section through the forebrain of an adult mouse showing the location of the subependyma (thick black lines) surrounding the lateral ventricles. The subependyma extends rostrally into the migratory pathway to the olfactory bulb. The total number of retrovirally labeled cells was counted within the subependyma from the crossing of the anterior commissure caudally and extending rostrally to include the olfactory bulbs. (B-E) Clones of retrovirally labeled cells (arrows) within the subependyma lining the lateral ventricle in an animal that received a kill (0-2-8 day; B,C) and a control animal (0-6 day; D,E). The 5 lv cells in the 0-2-8 day clone are located on the lateral wall of the ventricle in the same 60 μm section (B,C). This clone also contained 4 cells in the olfactory bulb (not shown). (D,E) The two cells shown in the 0-6 day lateral ventricle clone are in adjacent sections. This 0-6 day clone contained 2 additional cells in the olfactory bulb. Bar, 20 μm.

gene). To assure that this criterion was met, animals received bilateral injections on day 0 and were perfused 20 or 36 hours later (short survival times). Only 55% of the hemispheres injected with retrovirus contained any β-galactosidase-positive cells at 36 hours, this suggests that infection of a cell occurred in a one-or-none fashion at the dilution of retrovirus used in these studies. The average clone size was 2.3±0.4 cells and the cells were within 180 μm of each other. At 20 hours postinfection the average number of cells per clone is not significantly different from 36 hours (t_{32}=2.0, $P≥0.05$) yet only 34% of the injected hemispheres contained β-gal-positive cells. This suggests that the difference between the 55% infection rate after 36 hours and the 34% infection rate at 20 hours may be due to the fact that not all of the infected cells were expressing the gene product 20 hours postinfection. At both times, the clone size is greater than one since the infected cell

has divided at least once or twice (Tc=12.7 hours) in a steady-state mode of division during this time. After 2 rounds of division the maximum clone size would be 4 cells (based on symmetric division with no cell loss). Accordingly, a clone size of >4 cells (observed 1/18 times, 5.5%) must be due to a double infection (2 cells in a single hemisphere infected with the retrovirus) or to an infected cell with a shorter cell cycle time (see below). Our observed clone size of 2.3 cells after 36 hours is due to the combination of asymmetric division and cell death within the lineage of the constitutively proliferating cells. Thus, since there is no reporter gene turn-off in the subependymal cells in the lateral ventricle (Craig et al., 1998) and given the rate of infection and the size of the clones at 36 hours, we conclude that the cells observed at longer survival times (see below) are the progeny of a single retrovirally infected cell in each hemisphere.

One group of animals received retrovirus injections on day 0 and were perfused 6 days later (0-6 day animals). We chose a 6 day survival time to allow for a direct comparison of postretroviral infection survival times between this group and the group of animals that received high doses of [^{3}H]thy on day 0, a retrovirus injection on day 2 and were perfused 6 days later (see 0-2-8 day group below). In the 0-6 day animals the retrovirus will most likely infect a constitutively proliferating cell and not a stem cell since constitutively proliferating cells are by far the most mitotically active cells within the subependyma (Morshead et al., 1994). The 0-6 day group had an infection rate of 46% and an average clone size of 4.3±0.5 cells (significantly different from 0-36 hour controls, t$_{43}$=2.15, P<0.05). The slight decrease (non-significant) in infection rate after 6 days compared to 36 hours may reflect the loss (not reporter-gene turn-off) of entire clones in the adult subependyma that occurs during these 6 days and which would be even larger over longer survival times (Craig et al., 1998). As illustrated in Fig. 2B-C, the cells within the clones had the morphology of undifferentiated cells, some of which had single processes. This undifferentiated phenotype was observed in control animals as well as those that underwent the kill protocol.

The location of cells within each clone could be used to quantitatively determine the fates of the constitutively proliferating population. First, although 100% of all the cells observed at 36 hours were located within the subependyma surrounding the lateral ventricle (lv), this was not true of longer survival times where some cells were found in the olfactory

bulb (ob) (Table 1). At 6 days, 40% of the clones contained cells in the ob exclusively, 26% contained cells only in the lv and the remaining 34% of the clones had cells in both the lv and ob. Since 40% of the clones are entirely lost from the lv after 6 days, we suggest that this represents 40% of the life span of the constitutively proliferating population within the lv. Accordingly, the average survival time for a constitutively proliferating cell in the lv is 15 days. It is important to note that this estimated life span is strictly for the subependymal cells within the lv and is not an appropriate survival time estimate for cells en route to or within the olfactory bulb.

Second, the distribution of the cells within the clones revealed that on average, a single constitutively proliferating cell gives rise to 2.8±0.5 ob cells in 6 days and 1.6±0.4 cells remain in the lv. This observation is consistent with previous work showing that the size of the clones within the lv remains constant over time (Morshead and van der Kooy, 1992), since the average lv clone at 6 days (1.6±0.4 cells) is not significantly different from 36 hour controls (2.3±0.4 cells, t$_{43}$=1.3, P>0.05). Previous work also revealed that constitutively proliferating cells proliferate in a steady-state mode of division whereby one of the progeny continues to divide (Morshead and van der Kooy, 1992) with a cell cycle time of 12.7 hours. The other progeny undergoes cell death (Morshead and van der Kooy, 1992; Blaschke et al., 1998) or migrates to the olfactory bulb (Lois and Alverez-Buylla, 1994). Accordingly, we would expect to see at least 11 cells after 6 days if one of the progeny of each division stayed alive but did not continue to proliferate and assuming that host incorporation of the retroviral DNA occurs within 1 cell cycle (Roe et al., 1993). This expected value of 11 cells does not take into consideration cells with a different cell cycle time, since proliferation kinetics analyses strongly suggest single population dynamics within the subependyma (Morshead and van der Kooy, 1992). Since only 4.3 cells are observed after 6 days, a minimal estimate is that 60% of the progeny undergo cell death. The 40% that stay alive can be divided into two groups with 25% migrating to the bulb (2.8 ob cells out of 4.3 total cells observed times 0.4) and 15% confined to the lv (1.6 lv cells out of 4.3 total cells observed times 0.4). The 60% estimate of cell death is likely a minimal estimate for two reasons. First, Menezes et al. (1994) have shown that cells continue to proliferate en route to the bulb, therefore, as the migrating progeny continue to divide symmetrically more cell death will occur even within the 25% of migrating ob cells. Second, over time entire clones will

Table 1. Clone distribution and size reveal characteristics of subependymal cell kinetics in vivo

| Group | n | Infection rate | Location of cells within clone | | | Average size of clones | | | | | |
			lv only	ob only	lv & ob	lv only	ob only	lv & ob	all lv cells	all ob cells	all cells
0-36 hours	33	55%	100%	0%	0%	2.3±0.4	0	0	2.3±0.4	0	2.3±0.4
0-6 days	58	46%	26%	40%	34%	2.4±0.9	3.5±0.6	6.9±1.8	1.6±0.4	2.8±0.5	4.3±0.5
0-2-8 days	73	27%	45%	5%	50%	7.9±1.6	1.0	17.2±1.7	8.7±1.8	3.5±1.3	12.2±2.7

n = number of hemispheres injected with retrovirus.
lv only = clonally related retrovirally labeled cells found within the subependyma lining the lateral ventricle only.
ob only = clonally related retrovirally labeled cells found within the olfactory bulb and/or along the migratory pathway only.
lv & ob = clones with cells found in both the subependyma lining the lateral ventricles as well as within the olfactory bulb and/or along the migratory pathway.
all lv cells = average size of clones confined to the lateral ventricle only ('lv only') as well as any lv cells found within 'lv & ob' clones.
all ob cells = average size of clones confined to the ob only ('ob only') as well as any ob cells found within 'lv & ob' clones.
all cells = average clone size of all clones observed within this survival time group.

eventually die out (Craig et al., 1998; present work). Hence, 60% is a conservative estimate for the amount of cell death and fairly reflects the dynamics reported by others within the lineage.

Clone size increases by 2.8 times after repopulation of the subependyma

A strong prediction from previous work showing repopulation of the subependyma by stem cells after depletion of the constitutively proliferating population (Morshead et al., 1994) is that the average clone size should increase following repopulation compared to control animals which did not receive a kill (assuming that the total number of stem cells residing in the subependyma is less than the total number of constitutively proliferating cells). To test this prediction we killed the constitutively proliferating cells on day 0 using high doses of [^{3}H]thy followed by retrovirus injections on day 2, when 50% of the stem cells are proliferating (Morshead et al., 1994, 1997), and perfused the mice on day 8 (0-2-8 group; 6 days post-infection, similar to the control 0-6 day group). By injecting retrovirus on day 2 we increased the probability that we would infect a stem cell and thus see larger clones. The animals were examined at day 8 post-kill since this is the time necessary for complete repopulation of the subependyma (Morshead et al., 1994). It should be noted that on day 2 post-kill when 50% of the stem cells are proliferating there is also proliferation of a transit amplifying population (a transient population of symmetrically dividing cells) by way of which the constitutively proliferating cells are derived (Morshead et al., 1994; present study). Indeed, during repopulation we observed a 2.8 times increase in the size of the clones compared to the 0-6 day group (12.2±2.7 cells/clone in the 0-2-8 group versus 4.3±0.5 cells/clone in the 0-6 group, t_{45}=3.22, P<0.05; Table 1).

It is important for our interpretation of the 2.8 times increase in clone size that the kill paradigm did in fact deplete the constitutively proliferating subependymal cells. We performed scintillation counting on subependymal tissue samples from animals that were perfused 1 hour, 1 day and 2 days post-kill and observed a progressive loss in the amount of incorporated radioactivity within the subependyma over this time course (628.4±105.4 cts per minute/mg at t=1 hour, 270.9±21.6 cts per minute/mg at t=1 day, 89.0±18.0 cts per minute/mg at t=2 days). By 2 days post-kill the amount of radioactivity was not significantly different from background counts obtained from uninjected tissue samples (89.1±18.0 cts per minute/mg at t=2 days versus 77.5±21.2 cts per minute/mg background, t_2=0.6, P>0.05). This loss of radioactivity in the subependyma is not accounted for by migration of the labeled cells to the olfactory bulbs since we also observe a progressive decrease in the amount of radioactivity to background levels in the olfactory bulbs by 2 days post-kill. Therefore we conclude that proliferating cells that incorporated the high doses of [^{3}H]thy died.

The locations and distributions of the cells within the clones also differed markedly between the 0-6 day group and the 0-2-8 day group (Table 1). In the repopulation (0-2-8) group, 95% of all the clones still contained lv cells (compared to only 60% in 0-6 day animals), and thus only 5% of the observed clones in 0-2-8 day animals were found exclusively in the ob (1 cell observed in the ob of 1 hemisphere) compared to 40%

ob only clones observed in the 0-6 day animals. These data suggest either that fewer entire clones are migrating to the ob during the repopulation phase or that clones are more likely to maintain a cell within the lv during repopulation.

The cells within the larger clones tended to be clustered in the lv (Fig. 2B,C). A number of observations lead us to conclude that the larger clones are composed of undifferentiated cells similar to control clones and not of infected differentiated cells induced to proliferate after injury. First, since the retroviral gene product is expressed in the cytoplasm of infected cells it is easy to observe cell morphology and make the distinction between different cell types. After a kill, retrovirally labeled cells had the same undifferentiated morphology as cells in control clones and did not exhibit the morphology of astrocytes or oligodendrocytes or microglia (Fig. 2B-E). Indeed, in previous experiments when larger volumes of retrovirus were injected into the lateral ventricle we observed labeling of astrocytes and oligodendrocytes in the corpus callosum of control brains at short survival times (Morshead and van der Kooy, 1992). Presumably, the large volume of retrovirus (1.0 μl) infected damaged glial cells when it refluxed up the cannula injection pathway. We did not observe damaged astrocytes and oligodendrocytes with the smaller volume injected in the present experiments. Second, a group of animals received a kill on day 0 followed by 10 hours of BrdU labeling (5 injections, 1 every 2 hours) on day 2 post-kill which is the same time that the retrovirus was injected. The brains were processed for the presence of double-labeled cells using S100 to label astrocytes and ependymal cells and BrdU to assess the proliferating population after a kill. We observed no double-labeled cells suggesting that S100-positive glial cells were not proliferating within the subependyma 2 days post-kill (unpublished observations). These findings make it unlikely that the large clone size in 0-2-8 day animals is due to the infection of damage induced glial cells.

Repopulation of the subependyma does not occur at the expense of migration to the ob. While the size of the clones within the lv was significantly greater (5 times) after a kill compared to controls (8.7±1.8 lv cells/clone versus 1.6±0.4 lv cells/clone, respectively, t_{45}=4.4, P<0.05), the size of the ob clones was not significantly different between these same groups (3.5±1.3 ob cells/clone versus 2.8+0.5 ob cells/clone, t_{45}=0.6, P>0.05). Thus, during repopulation the absolute number of cells migrating to the ob is maintained despite the 5 times increase in the number of cells within the lv (Fig. 3).

Non-random clone size distribution suggests stem cell kinetics

We examined the relative frequencies of different clone sizes in the various treatment and survival time groups (Fig. 4) during repopulation of the subependyma. The vast majority of clones in the 0-36 hour group were 1-3 cells in size (Fig. 4A), as would be predicted from the 12.7 hour cell cycle time for the constitutively proliferating cells. Since the constitutively proliferating cells have divided at least twice and possibly 3 times in 36 hours, we could expect to see as many as 4 cells from asymmetric divisions. The progeny may be destined to migrate to the olfactory bulb or undergo cell death at a later time (all of the cells in this 36 hour group were observed in the lv). One of 18 clones contained 7 cells (5.5%), which may be
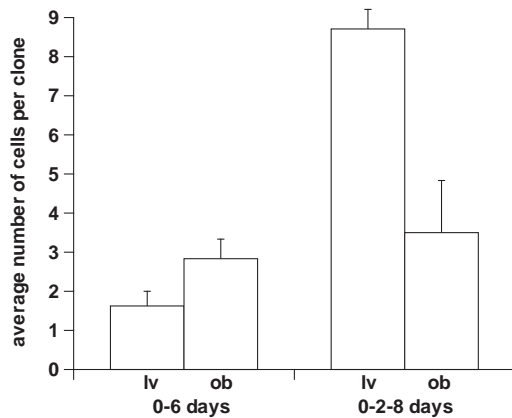
Fig. 3. The average number of retrovirally labeled cells per clone observed within the lateral ventricle (lv) and olfactory bulb (ob) in control animals (lv, $n=16$; ob, $n=17$) surviving for 6 days following retrovirus injection compared to animals that received a kill on day 0, retrovirus on day 2 and were perfused 6 days later (day 8) (lv, $n=19$; ob, $n=13$). The lv and ob values are composed of all cells found at these locations from all clones analysed (i.e. lv value includes all cells from 'lv only' clones as well as lv cells from 'lv and ob' clones). The average number of cells found within the lv of the repopulation group is 5 times larger than that in controls and can account for the entire increase in clone size observed following repopulation of the subependyma. There is no significant difference in the sizes of the ob clones between these same groups. Data represent means ± s.e.m.

the result of cells proliferating with a shorter cell cycle time or may be due to a double infection (two cells infected with the retrovirus in a single hemisphere).

The distributions of clone sizes varies dramatically in groups analysed 6 days after retroviral infection, under baseline conditions where a constitutively proliferating cell is infected (Fig. 4B) compared to during repopulation where a stem cell is more likely to be infected (Fig. 4C). A Wilcoxon test confirmed that the 0-2-8 day repopulation frequency distribution was significantly different (with higher values) from the 0-6 day control frequency distribution ($P<0.05$). During repopulation both constitutively proliferating cells and transit amplifying cells are proliferating and thus both can be infected with the retrovirus. The relative proportions of these constitutively proliferating and transit amplifying cells at the time of infection are unknown, however the small clones observed during repopulation likely reflect the infection of the constitutively proliferating cells (1-6 cell clones, as seen in 0-36 hour and 0-6 day groups). We suggest that the larger clones (7-15 cells/clone) indicate infection of transit amplifying cells and the largest clones, which constitute 15% of the repopulation group (1 clone of 28 cells, 1 clone of 31 cells and 1 clone of 51 cells) are the result of infecting a stem cell. The observation that these largest clone sizes do not occur under baseline conditions (0-6 group) and that the frequency of occurrence of these large clones exactly matches the frequency at which mice with long term survival times (31 days post-retroviral infection) have retrovirally labeled cells (see below), suggests that this subset of largest clones are the result of the infection of a stem cell. Even in the 0-6 day group there is evidence from the larger clones (1 clone of 14 cells, 1 clone of 19 cells) that under baseline conditions the constitutively

proliferating population may be replaced via a transit amplifying cell, although the frequency of their occurrence is similar to the estimated rate of a double infection (5.5%).

By examining the largest clones in the repopulation group at 0-2-8 days, we were able to make a minimal estimate of the number of stem cells present in the brain. We observed the largest clone to be 51 cells in size. The constitutively proliferating population makes up 10% of all the subependymal cells (Morshead and van der Kooy, 1992; unpublished observations), so it follows that if a single stem cell can give rise to 51 cells, then approximately 0.2% (10/51 cells) of all of the subependymal cells are stem cells. Given that the 51 cell clone occurred at the same frequency as the possible double infection rate, a more conservative estimate would use the 28 cell clone for the analysis, in which case we would estimate that approximately 0.4% of the subependymal cells are stem cells. These estimates assume that most of the stem cells are recruited during repopulation. Indeed, previous work showed that while 50% of all the stem cells were proliferating specifically on day 2 following depletion of the constitutively proliferating cells, the majority of stem cells were in fact recruited to repopulate the subependyma over the first 3 days post-kill. Morshead et al. (1994) showed that animals that received high doses of [³H]thy on day 0 to kill the constitutively proliferating cell and a second high dose of [³H]thy on day 2 to kill cells proliferating during the repopulation phase had a 50% reduction in the total number of neurospheres isolated in vitro, thus indicating that 50% of the stem cells were proliferating and hence killed on day 2. This 50% reduction exactly matched the 50% depletion in the number of constitutively proliferating cells observed in vivo following a second kill on day 2 with survival times of up to 31 days (Morshead et al., 1994). It follows that if the number of stem cells proliferating on day 2 reflected the total number of stem cells recruited, then a second kill on day 2 would have resulted in no reconstitution of the constitutively proliferating cells. Accordingly, many more than 50% (perhaps up to 100%) of the stem cells must have been recruited to repopulate the subependyma during the first 3 days after the initial kill on day 0 which depleted the constitutively proliferating cells.

The larger clone sizes following repopulation of the subependyma appear to clump in multiples of 7-9 (Fig. 4C). A Kolmogorov-Smirnov test for uniformity revealed that the 0-2-8 day data are not uniformly distributed (t=2.53, $P<0.05$). In other words the frequency distribution of clone sizes is not what one would predict if repopulation of the subependyma occurred in a random fashion. This observation of repeated doubling of clone size fits well with a simple model of stem cell proliferation during repopulation (Fig. 5). The model suggests that a single stem cell self-renews and gives rise to a cell which continues to proliferate. This cell continues to divide in a symmetrical fashion to amplify the stem cell division (thus, this is a transit amplifying cell). Eventually the progeny of these symmetrical divisions enter into an asymmetrical division pattern whereby only a single cell continues to proliferate and the other undergoes cell death or migrates to the ob (thereby becoming the constitutively proliferating population). This model can account for the sizes of clones observed after repopulation. A cell that was infected early in the lineage (a transit amplifying cell) would result in a large clone as a result of symmetrical divisions. A cell infected later

in the lineage would exhibit the constitutively proliferating cell mode of proliferation and hence result in smaller clones. The largest clones observed result from infection of stem cells.

Stem cells undergo asymmetric division during repopulation

Any adult stem cells that were infected with retrovirus should be present at much longer survival times since stem cells by definition, show long term self-renewal (Hall and Watt, 1989; Potten and Loeffler, 1990). The present data suggest that under baseline conditions the life span of the constitutively proliferating cell is in the order of 15 days, and other work has shown that by 28 days post-infection there are few retrovirally labeled cells present within the lv under baseline conditions (Craig et al., 1998). Accordingly, β-galactosidase-positive cells at survival times longer than 28 days are not constitutively proliferating cells, but instead probably infected stem cells. Thus, to test that we have retrovirally infected stem cells during repopulation, a group of animals received a $[^3H]$thy kill on day 0, retrovirus on day 2 and were perfused on day 33. These animals were compared to a control group which received retrovirus on day 0 and survived for 31 days. We observed 3/66 hemispheres (4.5%) with β-galactosidase-positive cells within the lv in control 0-31 day animals compared to 7/45 hemispheres (15.6%) in the 0-2-33 day group. Thus, it is 3.5 times more likely that a stem cell will be infected after a kill than under baseline conditions. Once again, all of the retrovirally infected cells were small, undifferentiated cells identical to those observed in control and kill animals. Interestingly, this 15% infection rate of stem cells is the same as the percentage of larger clones observed in the 0-2-8 day animals (15% of the clones had 28 cells or greater), and serves as independent confirmation that the largest clones observed result from the infection of a stem cell.

The average size of the clones within the lv was the same in both long survival groups (1.3±0.9 cells/clone in 0-31 day animals versus 1.4±0.2 cells/clone in 0-2-33 day animals, t_8=0.3, P>0.05, Fig. 6). All the clones in the lv were 1-2 cells in size at these long survival times. This suggests that the simple asymmetrical mode of division of the stem cell does not change even during the repopulation of the subependyma when it is making up for the 10% loss in the total number of subependymal cells after a kill.

At 33 days post-kill there is a small mean increase in the number of ob cells per clone compared to the control 0-31 day groups, however this difference does not reach statistical

significance (t_{20} =1.8. P>0.05, Fig. 6). The lack of a significant increase in ob cells post-kill is consistent with the observation at 8 days post-kill when there is no increase in the number of ob cells, but a significant increase in the number of lv cells. Recent work by Craig et al. (1998) indicates that at long survival times (28 days) there is significant turn-off of β-gal
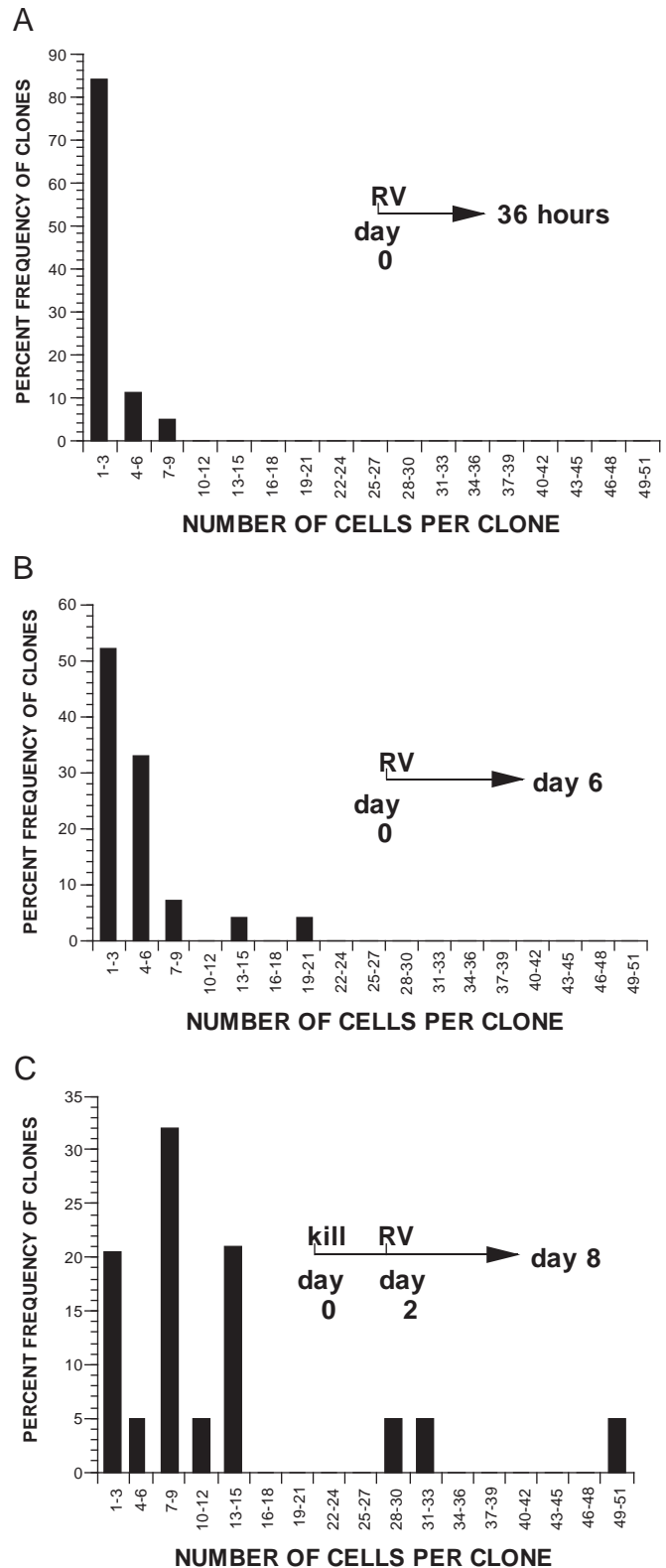
Fig. 4. The numbers of retrovirally labeled clones of different sizes are expressed as percent frequencies of the total number of clones in each group. (A) Animals were perfused 36 hours after receiving a retrovirus injection. The small clone size combined with the 55% infection rate indicates that single cell infections were occurring. (B) Animals received retrovirus on day 0 and were perfused on day 6, which gives post-retroviral infection survival times comparable to (C) in which animals received a kill on day 0, retrovirus on day 2 and were perfused 6 days later (day 8). Eight days is the time necessary for repopulation of the cp cells within the subependyma after a kill (Morshead et al., 1994). The average number of cells per clone increased by 2.8 times following repopulation (C) and the frequency distribution of clone sizes in this repopulation group reveals a repeated doubling in the sizes of the clones (multiples of 7-9 cells).
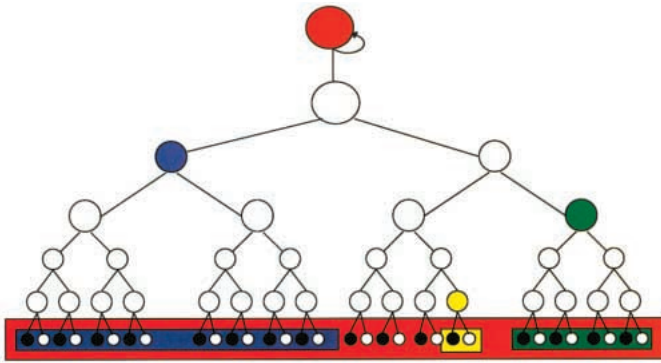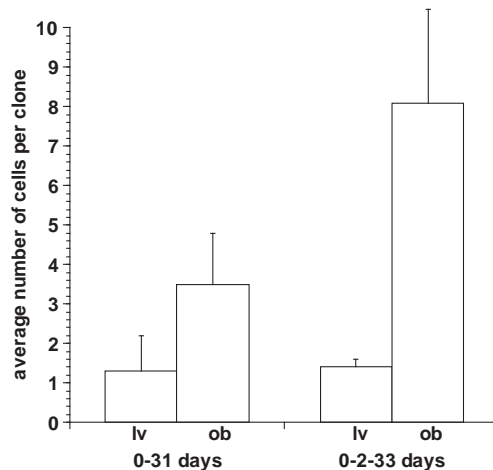
Fig. 5. The model shows that the variation of clone sizes following repopulation can result simply from the stage in the cell lineage that retrovirus infection occurred. For example, retrovirus infection of the green cell would result in an 8 cell clone whereas infection of the blue cell results in a 16 cell clone, etc. Small clones (1-2 cells) can be explained by infection of constitutively proliferating cells further along the lineage tree (yellow cell). This simple model indicates that repopulation of the constitutively proliferating cell population occurs by symmetrical division of a transit amplifying cell and does not involve cell death. Black cells represent those progeny of constitutively proliferating cells that are destined to undergo cell death or to migrate to the olfactory bulb, the normal fates of the constitutively proliferating cells in vivo. The single cell at the top of the lineage (red) represents a stem cell.

Fig. 6. The average number of cells in the lv of clones observed in control (0-31 days, n=3) animals and those that received a kill on day 0 (0-2-33 days, n=7). There is no significant difference between the sizes of the lv clones between these groups. The average number of cells observed in the ob of the same groups reveals a trend towards an increase in number, although the values are not significantly different (0-31 days, n=11; 0-2-33, n=20). The lv and ob values consist of all cells found at these locations from all clones analysed (i.e. lv value includes all cells from 'lv only' clones as well as lv cells from 'lv and ob' clones). Data represent means ± s.e.m.

gene expression specifically within the ob population and not within the lv. Accordingly, we have likely underestimated the total number of ob cells in both the kill and control group at this long survival time. Indeed, the turn-off of β-gal expression may be a source of error making the difference between the 0-31 day and 0-2-33 day survival time insignificant in the ob (Fig. 6). The increased numbers of retrovirally labeled cells in the lv 8 days (Fig. 3) compared to 33 days (Fig. 6) post-kill may represent the presence of both transit amplifying cells and constitutively proliferating cells at 8 days, both of which have a life span of less than 31 days. Only stem cells remain retrovirally labeled at survival times of 31 days or more.

An estimate of the total number of stem cells

Animals received injections of BrdU sufficient to label the entire constitutively proliferating population over its estimated cell cycle (5 injections, 1 every 2 hours; Morshead and van der Kooy, 1992). Control animals received BrdU on day 0 and were perfused 0.5 hours after the last injection. Two other groups of animals received a [³H]thy kill on day 0, BrdU injections on day 2 when 50% of the stem cells are proliferating (Morshead et al., 1994), and were perfused 0.5 hours or 31 days after the BrdU. By counting the total number of BrdU-labeled cells within the subependyma 33 days post-kill, the only labeled cells within the subependyma will be stem cells and thus, we could make an estimate of the total number of stem cells present within the subependyma. Animals that received a kill followed by BrdU injections on day 2 and were immediately perfused revealed a 76% reduction in the number of BrdU-labeled cells compared to control mice (no kill and immediate perfusion after BrdU) (43.0±5.1 cells/hemisection versus 178.6±13.4 cells/hemisection, respectively). This is comparable to previous work showing that 2 days post-kill the number of proliferating

cells is decreased 72% compared to controls (Morshead et al., 1994). Importantly, this reduction in proliferation after a kill is mirrored by a considerable decrease in the overall retroviral infection rate post-kill, although the clone size which results post-kill is 2.8× larger than controls. To calculate the total number of stem cells, the absolute number of BrdU-labeled cells was counted in the 33 day survival animals surrounding both ventricles in every 10th section from the rostral tip of the crossing of the corpus callosum to the rostral tip of the crossing of the anterior commissure, and an average number of cells was obtained per section. This average number of cells (3.4±0.8 cells per hemisphere ×2 = 6.8 cells per section) was multiplied by the total number of sections within the defined brain region (91 sections). Multiplying the total number of BrdU-labeled cells by the total number of sections gives a value of 618.8 cells, which represents 50% of the total number of stem cells, since BrdU was injected on day 2 post-kill when 50% of the stem cells are proliferating (Morshead et al., 1994). Thus, the absolute number of stem cells within the defined region of the adult forebrain is approximately 2×618.8=1238 cells. This value represents 0.4% of the total number of subependymal cells (1238 stem cells/304,900 total Nissl-stained subependymal cells) in the same region (Morshead and van der Kooy, 1992). This 0.4% value is similar to and supportive of the estimate of the percentage of stem cells obtained from analysing the largest clone sizes observed after a kill, which was that 0.2-0.4% of all of the subependymal cells were stem cells.

DISCUSSION

The present work examines the kinetics of the multipotential stem cell and its progeny the constitutively proliferating cells

within the subependyma of the adult mammalian forebrain. A clonal analysis was undertaken to examine characteristics of these populations, (1) under baseline conditions and (2) following reconstitution of the subependyma after high doses of [³H]thy were used to kill the constitutively proliferating cells (Morshead and van der Kooy, 1994). This clonal study has revealed that the constitutively proliferating cells have an average life span of 15 days within the lateral ventricle subependyma. Examining the sizes and distributions of the clones under baseline conditions 6 days following retrovirus infection revealed that on average 60% of the cells undergo cell death, one of the previously described fates of the constitutively proliferating cells (Smart, 1961; Morshead and van der Kooy, 1992). Of the 40% of the cells that survive, 15% are found in the lateral ventricle subependyma and the remaining 25% are within the olfactory bulb (including the rostral migratory stream; Lois and Alvarez-Buylla, 1994). The neural stem cells, which reside in the lateral ventricle walls, can repopulate the constitutively proliferating population following its depletion with high doses of [³H]thy (Morshead et al., 1994). A strong prediction from this work is that the average size of a clone during repopulation would be larger than under baseline conditions since steady-state division of the constitutively proliferating population would not account for the increase in size after a kill. Indeed, injecting retrovirus on day 2 post-kill increased the probability of infecting a stem cell by 3.5 times and resulted in clones which were 2.8 times larger on average. Comparing the relative sizes of clones under baseline conditions (constitutively proliferating cell clones) and following repopulation of the subependyma after a kill (stem cell, transit amplifying cell and constitutively proliferating cell clones) enabled the construction of a simple model to explain the observed clone sizes (Fig. 5). This model can account for the observed doubling of clone sizes through retroviral labeling of proliferating cells at different points in the lineage tree as well as make predictions about the frequencies at which the different clone sizes will occur. An analysis of clone distribution at 6 days post-infection revealed that 40% of the clones were entirely confined to the olfactory bulb, with 34% having cells in both the lateral ventricle and olfactory bulb. Interestingly, this data shows that while 6 days is enough time to migrate to the olfactory bulb, not all cells within the subependyma behave in the same manner. The estimate of a 15 day life span for cells within the lateral ventricle subependyma is directly related to the percentage of clones observed within the olfactory bulb only at 6 days (40%). One prediction is that if we examine the distribution of clones 12 days postinfection, the 34% mixed clones at day 6 post-infection would all become olfactory bulb only clones. Accordingly, 74% of the clones would be olfactory bulb only clones at 12 days and the predicted life-span would be 16 days, very close to the value obtained from the 6 day analysis.

Given the 15 day life span for constitutively proliferating cells within the subependyma lining the lateral ventricles and the concomitant maintenance of the size of the subependyma throughout adult life (Tropepe et al., 1997), an estimate for the cell-cycle time (Tc) of the multipotential stem cell is 15 days. This is a minimum estimate of stem cell Tc, which results if the loss of a single constitutively proliferating cell from the lateral ventricle subependyma induces the asymmetric proliferation of a single stem cell to self-renew and to give rise

directly to a constitutively proliferating cell. However, there are two reasons to believe that this mode of replacement is not the case in vivo. First, the ratio of stem cells to constitutively proliferating cells is not 1:1 (Morshead et al., 1994; present study) and if there are more constitutively proliferating cells than stem cells, then the stem cell would need to proliferate with a much shorter Tc to replenish the constitutively proliferating cells in this direct fashion. Indeed, the two independent estimates from the present study of the number of stem cells within the subependyma indicate that the multipotential stem cells constitute <1% of the total subependymal population. Second, there is evidence from the larger clone sizes observed following repopulation (0-2-8 day clones) as well as in control 0-6 day animals that transit amplifying cells exist. These cells (Potten and Loeffler, 1990) are a transient population of dividing cells that amplify the one asymmetrical non-stem cell progeny of the stem cell to give rise to a larger number of differentiated cells (the constitutively proliferating cells in the present case). Thus, a transit amplifying cell would greatly minimize the amount of stem cell proliferation necessary to replenish the constitutively proliferating cells in the subependyma. Given these observations the estimate of a 15 day Tc for the stem cell can only be a rough approximation.

Further support for the estimate of 60% cell death comes from recent work showing that infusion of a cell survival factor (N-acetyl-L-cysteine) (Mayer and Noble, 1994; Henderson et al., 1996) into the adult lateral ventricle inhibits the naturally occurring cell death (Morshead and van der Kooy, 1996). Indeed, 5 days of infusion of N-acetyl-L-cysteine increases the total number of retrovirally labeled cells within the subependyma surrounding the lateral ventricles by 5 times over saline infused controls with no concomitant change in the number of cells found within the olfactory bulb (Morshead and van der Kooy, 1996). A simple interpretation of this 5-fold increase is that the cell survival factor is keeping the normally dying progeny of the constitutively proliferating cell within the lv alive. For example, over 5 days we would expect to see a minimum of 10 cells per clone during steady-state division (9 cell cycles in 5 days). The 5-fold increase in the average lv clone size means that 75% of the cells in the 10 cell clone (15% normally found in the lv times 5) were seen in the lateral ventricle subependyma with no change in the numbers of cells found in the ob (25%, as observed under baseline conditions). Hence, the observed clone size following infusion of N-acetyl-L-cysteine can account for the entire predicted clone size (10 cells) simply by keeping the normally dying progeny alive. These findings support the hypothesis that on average, at least 60% of the cells within a given clone undergo death.

Two independent methods were used to estimate the total number of stem cells present in the adult subependyma, and both estimates gave remarkably similar results (0.2% versus 0.4% of adult subependymal cells). Injection of BrdU on day 2 post-kill when 50% of the stem cells are proliferating (Morshead et al., 1994) provided a means of estimating 31 days later that 0.4% of the subependyma were stem cells. This estimate was based on counts of BrdU-labeled cells from a specific brain region (from the crossing of the corpus callosum rostrally to the crossing of the anterior commissure caudally) where cell cycle kinetics have been analysed in detail (Morshead and van der Kooy, 1992). Estimating the percentage

of stem cells from the clonal analysis using retroviral labeling also gave a value of 0.4% of adult subependymal cells. This value may be a slight overestimation as it targets the 28 and 31 cell clones observed in the 0-2-8 day group as stem cell clones (and not the 51 cell clone which was the largest clone observed following repopulation). Using the 51 cell clone for the analysis results in a stem cell estimate of 0.2% of adult subependymal cells. Several findings suggest that the largest clones observed following repopulation are not the result of infecting transit amplifying cells: (1) the largest clone sizes (≥28 cells) occur at the same frequency (15%) as the probability of infecting a stem cell on day 2 post-kill (15% of the hemispheres have clones at 33 day survival times); (2) the largest clones observed following repopulation (0-2-8 days) do not appear under baseline conditions (0-6 days); and (3) regardless of the methods used, the estimates of stem cell frequency in the adult forebrain subependyma are strikingly similar (0.2-0.4%).

One of the cardinal properties of a stem cell is that it is capable of long-term self renewal (Hall and Watt, 1989; Potten and Loeffler, 1990). Indeed, the present clonal analysis in the adult murine forebrain in vivo reveals single cell clones at long survival times in control mice (0-31 day) as well as in animals following repopulation of the subependyma (0-2-33 day). Single cell clones reveal that the stem cell proliferates asymmetrically to self-renew and produce a transit amplifying cell, under baseline conditions as well as during recruitment and repopulation of the subependyma. This in vivo result contrasts with the mode of division which is observed in vitro when adult forebrain stem cells are isolated in the presence of EGF or basic fibroblast growth factor (bFGF). In vitro, stem cells give rise to clonally derived spheres containing hundreds of cells (Reynolds and Weiss, 1992; Gritti et al., 1996). When the spheres are dissociated into single cells and replated in the presence of EGF or bFGF a single sphere gives rise to multiple spheres (Reynolds and Weiss, 1996; Gritti et al., 1996; Weiss et al., 1996), indicating that the stem cell went through multiple symmetric divisions producing many stem cell progeny. The difference in stem cell kinetics in vivo and in vitro suggests either, (1) the presence of inhibitory constraints in vivo which limit the symmetric self-renewal of the stem cell (for example, cell-cell contact) or (2) the absence of a positive signal in vivo to induce the symmetric division. Interestingly, in vivo infusion of EGF into the lateral ventricles resulted in a 3.7-fold increase in the total number of stem cells isolated in vitro (Craig et al., 1996). This observation lends support to the hypothesis that a positive signal inducing symmetrical stem cell division may be lacking in vivo.

Considerable work has examined the migration of cells to the olfactory bulb and revealed that the most rostral subventricular zone gives rise to the neural precursors destined for the ob (Luskin, 1993; Lois and Alvarez-Buylla, 1994). We compared the locations in the lateral ventricle subependyma of retrovirally labeled cells, which were present 31 days after retrovirus infection, in clones that contained lateral ventricle subependyma cells only and in clones that contained lateral ventricle subependyma and olfactory bulb cells. Subependymal cells within the lateral ventricle only clones had a greater tendency to be more caudally located (an average of 300 μm rostral to the anterior tip of the crossing of the anterior commissure) compared to the location of lateral ventricle cells

in the clones that contained lateral ventricle subependyma and olfactory bulb cells (an average of 540 μm rostral to the anterior tip of the crossing of the anterior commissure). This difference suggests that the distribution of the stem cells along the rostrocaudal axis is caudally biased relative to the distribution of neural precursors, producing progeny that will migrate into the olfactory bulb. Looking at the distribution of stem cells within the subependyma (as assayed by BrdU labeling 31 days after injection) revealed that only 12% of the stem cells were located in the dorsolateral corner of the lateral ventricle (unpublished observations), the location where neural precursors destined for the olfactory bulb reside (Luskin, 1993; Lois and Alvarez-Buylla, 1994). Taken together, the evidence suggests that the distribution of stem cells within the adult lateral ventricle subependyma is somewhat different from that of the neural precursors producing olfactory bulb neurons, and that the two populations are not homogeneously intermixed.

The absolute number of retrovirally labeled cells which migrated to the olfactory bulb was not significantly different in animals that did or did not receive a [^{3}H]thy kill. Indeed, the entire increase in clone size seen 8 days post-kill can be accounted for by an increase in the number of cells within the lateral ventricle (a 5 times increase in the number of retrovirally labeled lateral ventricle subependymal cells). The stem cells are recruited to proliferate and make up for a 10% loss in the total number of subependymal cells after a kill, yet there is maintenance of migration to the olfactory bulb over this same time. Furthermore, it is interesting that regardless of the survival time after retrovirus infection (i.e. 6 or 31 days), a single constitutively proliferating cell gives rise to the same number of olfactory bulb cells (3.8±0.5 versus 3.5±1.3 cells, respectively). One might have predicted that the 6 day clone would have 40% of the number of cells that the 31 day clones (6 days is 40% of the 15 day life span within the lateral ventricle of a constitutively proliferating cell). These data suggest that a mechanism exists for maintaining the absolute number of cells migrating to the olfactory bulb. Lois et al. (1996) have recently shown that cells migrate in a well-defined stream to the olfactory bulb, where cells within the chain use one another as a migration substrate. One possibility to explain the conserved absolute number of olfactory bulb cells despite dramatic changes in the total number of subependymal cells (decreased after a kill) is that the chain of migrating cells is physically restrictive in the adult and can only sustain the migration of a specific number of cells at any given time.

In summary, the behavior of adult mammalian forebrain stem cells and their progeny, the constitutively proliferating cells, have been clonally analysed in vivo. The cell cycle time of stem cells in vivo is unknown, thereby making the lineage analysis of this population a formidable task. We took advantage of an earlier finding that 50% of the stem cells in vivo are proliferating 2 days after the depletion of the constitutively proliferating cells (Morshead et al., 1994). Accordingly, injections of retrovirus at this time greatly enhance the probability of infecting a stem cell. The analysis has revealed that under baseline conditions 6 days after labeling, 25% of the progeny of the constitutively proliferating cells migrate to the olfactory bulb, 15% remain confined to the subependyma surrounding the lateral ventricle and 60% undergo cell death. The average survival time of a constitutively proliferating cell within the lateral ventricle is 15

days and we suggest that 15 days may be a rough approximation of the cell cycle time of the stem cells in vivo under baseline conditions. We estimate that approximately 1,200-1,300 stem cells are present in the forebrain (between the anterior tip of the corpus callosum rostrally and anterior tip of the anterior commissure caudally). The stem cells constitute 0.2-0.4% of the subependymal population and proliferate in an asymmetric mode of division to self-renew and give rise to the constitutively proliferating population via transit amplifying cells.

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