EPENDYMAL STEM CELLS DIVIDE ASYMMETRICALLY AND TRANSFER PROGENY INTO THE SUBVENTRICULAR ZONE WHEN ACTIVATED BY INJURY

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Abstract—Evidence is presented to show that cells of the ependymal layer surrounding the ventricles of the mammalian (rat) forebrain act as neural stem cells (NSCs), and that these cells can be activated to divide by a combination of injury and growth factor stimulation. Several markers of asymmetric cell division (ACD), a characteristic of true stem cells, are expressed asymmetrically in the ependymal layer but not in the underlying subventricular zone (SVZ), and when the brain is treated with a combination of local 6-hydroxydopamine (6-OHDA) with systemic delivery of transforming growth factor-alpha (TGFα), ependymal cells divide asymmetrically and transfer progeny into the SVZ. The SVZ cells then divide as transit amplifying cells (TACs) and their progeny enter a differentiation pathway. The stem cells in the ependymal layer may have been missed in many previous studies because they are usually quiescent and divide only in response to strong stimuli. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

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Mitotic cells in the subventricular zone (SVZ) of the adult mammalian forebrain have generally been considered to be neural stem cells (NSCs) because throughout life they produce progeny that migrate along the rostral migratory stream (RMS) and differentiate into olfactory bulb neurons (Curtis et al., 2007; Goldman and Luskin, 1998). SVZ cells have been shown to be self-renewing and multipotent (Doetsch et al., 1999), but this is not sufficient to identify them as NSCs since the immediate downstream derivatives of adult stem cells (transit amplifying cells or TACs) may also be self-renewing and multipotent. Evidence from model systems indicates that a much more distinctive feature of authentic stem cells is asymmetric cell division (ACD), which allows the stem cell to replace itself at the same time as generating a daughter cell that enters a tissue-specific differentiation pathway (Betschinger and Knoblich, 2004; Wodarz and Huttner, 2003).

Here we show that in the forebrain of adult rats the cellular asymmetry characteristic of ACD is seen in the ependymal layer and not in the SVZ. Since ependymal cells are largely quiescent in vivo (Johansson et al., 1999) and some are probably post-mitotic (Spassky et al., 2005) we investigated their properties in an animal model of Parkinson’s disease (PD-model) where previous studies have shown a massive induction of cell proliferation and migration in response to a combination of lesioning with 6-hydroxydopamine (6-OHDA) and infusion of transforming growth factor (TGFα), referred to as PD-activation. (Cooper and Isacson, 2004; Fallon et al., 2000). We find that when activated by this combination of stimuli, ependymal cells divide both symmetrically to self-renew and asymmetrically to transfer progeny into the SVZ, where additional cell division occurs. The asymmetric divisions produce groups of adjacent cells that are coordinated in the cell cycle and clonally related. We also show that ependymal cells may be neurogenic in vivo since they give rise to SVZ cells that express a neuronal marker. We conclude that ependymal cells are the true NSCs of the mammalian brain whereas SVZ cells are their transit amplifying progeny, consistent with the biology of stem cells predicted by model systems.

EXPERIMENTAL PROCEDURES

Adult male rats at least 10 weeks of age were given PD-activation using the previously published protocol (Fallon et al., 2000). A solution of 6-OHDA was prepared immediately before injection from 4.8 mg/ml of 6-OHDA in 0.9% saline with 0.01% ascorbic acid. The rats were injected with 8 μl at a rate of 1 μl/min at the rostral border of the substantia nigra ventral-tergental area using a Kopf stereotactic device (David Kopf Instruments, Tujunga, CA, USA) (−5.3 A/P, −2.0 M/L, −7.9 D/V). The TGFα (R&D Systems Inc., Minneapolis, MN, USA) solution was prepared with 100 μg of TGFα in 200 μl of PBS and used to fill Alzet osmotic minipumps (ALZET Osmotic Pumps; DURECT Corporation, Cupertino, CA, USA) which were then incubated overnight in saline at 37 °C. The minipumps were stereotaxically implanted into the left caudate-putamen (±0.5 A/P, −5.3 M/L), and the infusion was delivered to the ipsilateral forebrain for the duration of the experiment. For the lentivirus experiments animals were injected 2 weeks prior to PD-activation with 250,000 virus units (America Pharma Source) in 10 μg/ml polybrene in the lateral ventricles at the coordinates (−1.1 A/P, +1.3 M/L, −4.9 D/V). BrdU was administered intraperitoneally at 50 mg/kg at specific times, and then the animals were killed at four time points. Animals for the 24 h time point received a single injection of BrdU at the time of surgery, and a subgroup of these animals was allowed to survive for an additional
3 or 5 days to establish a BrdU-chase period. Animals for the 5, 7 and 28-day time points received a single injection of BrdU 24 h before kill. The animals were then killed by perfusion with saline and fixed with 4% paraformaldehyde, the brains removed and post-fixed in the same solution overnight. Following post-fixing the brains were cryo-protected overnight at 4 °C in 30% sucrose.

Prior to sectioning, the perfused brains were embedded in OCT compound and coronal sections were cut at 20 μm on a −20 °C cryostat. The sections were immediately mounted on slides and stored at −20 °C. Sections were permeabilized in 0.1% Triton X-100 for 30 min at room temperature, then blocked in 5% goat serum in 0.1% Triton X-100 for 1 h at room temperature. Primary antibodies were dissolved in blocking solution at a 1:100 dilution then used to incubate the tissue sections overnight at 4 °C. The sections were subsequently washed in 0.1 M PBS and incubated in fluorescent secondary antibody at a 1:250 dilution, washed and counterstained with DAPI, then mounted on slides with Vectashield. In sections stained for BrdU incorporation the DNA was first denatured in 50% formamide in 2 SSC for 1 h at 65 °C, then the sections were washed in 2 SSC and incubated in 2 M HCl at 37 °C for 30 min. Finally the sections were washed in 0.1 M boric acid at pH 8.5 for 10 min and then washed in 0.1 M PBS. The tissue was evaluated immunohistochemically using antibodies recognizing stem-cell markers Nestin (Abcam, Cambridge, MA, USA), Vimentin (Invitrogen Corporation, Carlsbad, CA, USA), ependyma markers, CD24 (Becton Dickinson), s100β (Invitrogen), candidate ACD determinants aPKCζ, Gai (Santa Cruz), LGN (Dr. Thomas Chappell), Numb (Abcam), proliferation markers BrdU (Abcam), Histone H3 phospho-serine 10 (Upstate, Abcam), and differentiation markers GFAP (Invitrogen), βIIIITubulin (Millipore [Chemicon], Temecula, CA, USA). Stained tissue was imaged on a Zeiss LSM510-META microscope using two-photon excitation of DAPI counterstains and conventional confocal for all other colors. Histone H3-phospho-Ser-10 positive cells were counted within the ependymal layer and SVZ and ependymal cells were identified with apical markers of the surface and by checking for nuclei interposed between the Histone-H3 positive cells and the ventricle lumen. In cases where ependymal identity was in doubt the cell was counted as a SVZ cell. Standard errors were calculated using the number of animals at each time point, n=3 except at 28 days where n=4. Data were plotted using Excel and t-tests calculated using Minitab to make pair-wise comparisons to the control. Mitotic spindle angles were measured using a protractor. Images of the lentivirally labeled clones were captured as z-stacks and then reconstructed in Adobe Photoshop by applying images that were adjusted for pixel range and contrast. The three dimensional reconstructions were made in Bitplane-Imaris software.

RESULTS

Ependymal cells express ACD markers

Several protein markers of ACD are strongly expressed and asymmetrically localized in the ependymal layer, whereas in the SVZ they are either weakly expressed or

Fig. 1. Protein markers of the ependymal layer. (a–c) ACD proteins Gai, LGN, and PKCζ are apico-laterally localized on ependymal cells and face the ventricle. Numb marks the basal surface of the ependymal layer in b. (d–f) Apical ACD proteins are co-localized with the ependymal markers CD24 and s100β. (g) ACD protein Numb is baso-laterally localized on the ependymal layer and is asymmetrically localized relative to Gai and LGN in b. (h) NSC marker Nestin is strongly expressed and apico-laterally localized on the ependymal layer but not in the SVZ. (i) Vimentin is also strongly expressed and apico-laterally localized on the ependymal layer.
not expressed, depending on location and conditions, and in the SVZ they show no evidence for asymmetric localization. Thus the G protein G\textsubscript{i}, which is usually associated with receptors at the plasma membrane (Koelle et al., 2006) shows clear localization in cortical crescents on the apical face of the ependymal layer (Fig. 1a, d, g). Its binding partner LGN, a homolog of Drosophila pins (Bellaiche et al., 2001) shows a similar apical localization (Fig. 1b, e). A typical protein kinase C-zeta (aPKC\textsubscript{z}) (Izumi et al., 1998) shows apical localization in both the cytoplasm and cortex (Fig. 1c, f) and is concentrated in the apicolateral region of many cells, suggesting localization at cell junctions similar to its association with tight junctions in epithelial cells (Izumi et al., 1998). Accepted markers of the ependymal layer, CD24 and S100\textsubscript{β} (Spassky et al., 2005) are co-localized with apical ACD markers at the surface of the ventricle (Fig. 1d–f). In sharp contrast, Numb shows distinct baso-lateral localization (Fig. 1b, g) often forming a thick basal layer beneath ependymal cells and clearly delineating their lateral boundaries.

The ependymal layer but not the SVZ also shows expression of several generally accepted NSC markers, including Nestin which sometimes shows apical localization on ependymal cells, consistent with its asymmetric distribution in NSCs in vitro (Fuja et al., 2004) (Fig. 1h). The related intermediate filament protein Vimentin which is expressed in close association with Nestin during neurogenesis (Frederiksen and McKay, 1988; Stagaard and Mollgard, 1989; Frisen et al., 1995) also shows apico-lateral localization, and like Nestin is weakly or not expressed in the SVZ (Fig. 1i). The hematopoietic stem-cell marker CD133/prominin-1 was recently reported to be specific to some ependymal cells (Coskun et al., 2008) and has been shown to be apically localized on neuro-epithelial cells in the developing ventricular zone (Kosodo et al., 2004).

**Activation of the ependymal layer in a PD model**

Chemical lesioning of the substantia nigra by 6-OHDA combined with striatal infusion of TGF\textsubscript{α} results in a massive response in the adult brain (Cooper and Isacson, 2004; Fallon et al., 2000) including an increase in the number of SVZ cells incorporating BrdU (Cooper and Isacson, 2004) and subsequent migration of a band of cells into the striatum where they differentiate into astrocytes and neurons (Fallon et al., 2000). We used this PD-model to investigate cellular proliferation in the ependymal layer and SVZ over the course of 28 days and counted over 1600 mitotic cells from 16 rats. The response to activation was bimodal, with the average number of histone H3-phospho-Ser 10 (HH3)-labeled cells per section increasing on both the treated and untreated sides of the brain in the first 24 h, although not to statistically significant levels (Fig. 2). The number of HH3-labeled cells per section decreased on both sides of the brain for the remainder of the first week but then increased substantially on the treated side (Fig. 2). This bimodal response was confirmed by assays for

![Average HH3 Cells per Section](image_url)

**Fig. 2.** Average number of histoneH3-labeled cells per section. The number of labeled cells increased on both the treated and untreated sides of the brain in the first 24 h after surgery and then decreased during the remainder of the first week. By 28 days the average number of labeled cells increased to statistically significant levels on the treated side but not on the untreated side. Data are presented as mean ± S.E.M. P values < .1 compared to the saline control were considered statistically significant, 28d Treated Side SVZ + E (P = .072), indicated by asterisk.
BrdU incorporation which also showed an initial increase, then a decrease, followed by a significant increase on the treated side (data not shown). After 28 days the number of HH3-labeled cells on the treated but not the untreated side exceeded prior levels and this increase was statistically significant compared with the saline control (Fig. 2).

**Ependymal cells divide asymmetrically**

Of 119 HH3-positive ependymal cells that were found, 10 were in anaphase of mitosis and provided direct evidence of proliferation in the ependymal layer. These mitotic ependymal cells were oriented at a range of angles relative to the ventricle surface and appeared to maintain the polarized distribution of apical and basal determinants as they went through mitosis. Ependymal cells dividing with the spindle axis at an oblique angle or perpendicular to the ependymal layer are predicted to preferentially distribute apically localized determinants to the apical cell (Fig. 3a, b, d, e) and basally localized determinants to the basal cell (Fig. 3c). Ependymal cells dividing with the spindle axis parallel to the layer are predicted to distribute apically and basally localized determinants to the daughter cells symmetrically (Fig. 3f). Measurement of the mitotic spindle angles showed that lower angles were more common than steeper angles with five mitotic spindles oriented at less than 30° from horizontal, and only one over 60° (Fig. 3g).

**Ependymal cells proliferate in coordination with SVZ neighbors**

When BrdU was administered at surgery and followed by 3 days of label-free chase, scattered patches of labeled cells were found, each including one or a few labeled ependymal cells associated with a group of labeled SVZ neighbors (Fig. 4) in a largely unlabeled background. These proliferative units were often pyramidal in shape with an ependymal cell at the apex and SVZ cells making up the base (Fig. 4a–d). When BrdU was administered at surgery and then followed by 5 days of label-free chase, similar cell groups were found but with less intense BrdU-labeling in the SVZ compared with the result after a shorter chase period.

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**Fig. 3.** Dividing ependymal cells. (a) The surface of the ependymal layer is delineated by staining with CD24 and the mitotic spindle of this ependymal cell is oriented at a 65° angle relative to the ventricular surface. The apical daughter remains within the ependymal layer while the basal daughter is positioned in the SVZ. (b) Here the apico-lateral ACD marker Gui marks the surface of the ependymal layer and the mitotic spindle is at a 45° angle relative to the ventricular surface. Gui is preferentially distributed to the apical daughter of the division while the basal daughter inherits none. (c) Here the mitotic spindle is at a 41° angle and the basal daughter preferentially inherits Numb as it moves into the SVZ. (d) Apically localized LGN is preferentially distributed to the apical daughter of a division in which the spindle axis is oriented at a 45° angle relative to the ventricular surface. (e) Here the mitotic spindle is parallel to the surface of the ventricle at a 15° angle but the basal daughter is still displaced into the SVZ. (f) The spindle axis of this mitotic ependymal cell is parallel to the ventricle surface and both daughters inherit Nestin equally. (g) Chart showing the distribution of spindle axis angle found among 10 dividing ependymal cells.
suggesting slower cell cycle speeds in the ependymal layer compared with SVZ cells (Fig. 4b). A three-dimensional reconstruction of the cell group in Fig. 4b showed that it was conical with adjacent SVZ cells on one side of the apical ependymal cell and spread out laterally in the plane of the section (Fig. 4c). Many similar BrdU-labeled groups were found lining the ventricles on both sides of the brain, and the ependymal cells of these groups were apical to and outside of the GFAP-positive SVZ (Fig. 4d). Staining for GFAP also revealed a SVZ cell that contacted the ventricle via an apical process, and this cell type was clearly distinguishable from the apical ependymal cells of the conical groups described here (Fig. 4d).

**SVZ cells are clonally derived from ependymal cells**

To test for a clonal relationship among cells within the BrdU-labeled proliferative units, animals were given an i.v. injection of lentivirus constitutively expressing green fluorescent protein (GFP), subjected to the PD-activation 2 weeks later and then injected with BrdU 24 h prior to kill at the 28-day time point. The ventricle walls of these animals showed many GFP-labeled groups of cells (at least 12 in two different brains), that extended from the ependymal layer into the SVZ and that were co-labeled with BrdU, similar to the groups found in the pulse-chase experiments (Fig. 5a). In contrast to the pulse-chase experiments, however, ependymal cells in these groups were weakly labeled with BrdU compared with their SVZ daughters, suggesting that their cell cycle speed at 28 days remained slower than in the SVZ (Fig. 5b, h). Staining with CD24 confirmed that the apical-most cells were ependymal cells immediately adjacent to the ventricle surface (Fig. 5c, e, f), and BrdU labeling showed the groups had a pyramidal shape characteristic of the proliferative units found in the earlier pulse-chase experiments (Fig. 5b, c, f). One group of cells co-labeled by GFP and BrdU was relatively elongated compared with the others and appeared to contain three pairs of daughter cells in the SVZ that were increasingly separated from one another with greater displacement into the SVZ (Fig. 5h–j). Staining with βIIIITubulin showed that the
most basal cells expressed this neuronal marker and that the cells immediately above them were positioned at the boundary where βIII Tubulin expression appeared (Fig. 5, k, l).

DISCUSSION

Ependymal cells are largely quiescent under normal conditions, and this may have caused them to be overlooked in previous studies of adult NSCs except for a few notable reports (Coskun et al., 2008; Johansson et al., 1999; Xu et al., 2005). However, in the PD-model investigated here (Fallon et al., 2000) there is significantly increased proliferation on the treated side of the brain 28 days after surgery, and this response enabled us to document the behavior of ependymal cells in a way that was previously impossible. The significant increase in cellular proliferation on the treated but not the un-
treated side of the brain is consistent with prior reports (Cooper and Isacson, 2004; Fallon et al., 2000) and we presume it reflects the greater availability of TGFβ on the treated side.

The fact that several markers of ACD are expressed asymmetrically in the ependymal layer but not in the SVZ strongly supports the idea that the ependymal layer contains the true NSCs. In Drosophila neuroblasts undergoing ACD, the homologs of Gxi, aPKCs, and LGN, which we find localized on the apical side of ependymal cells facing the lumen of the ventricle (Fig. 1a–f), are all apically localized while Numb, which we find baso-laterally, is localized basally (Fig. 1b, g) (Roegiers and Jan, 2004). Given this polarity, ependymal cell divisions that are oriented with the spindle at an oblique angle or perpendicular to the layer are examples of ACD in which the daughter inheriting the apical complex remains within the ependymal layer as a stem cell while the basal daughter inherits Numb and enters the SVZ where additional proliferation and migration occur (Fig. 3a–c). Although we have not yet found definite cortical localization of alternative determinants on opposite poles of individual dividing cells as has been reported for Drosophila neuroblasts (Wodarz and Huttner, 2003) the clear molecular asymmetry of the ependymal layer and of dividing ependymal cells is consistent with ACD exactly as predicted from studies on model systems. In addition about half of the ependymal cell divisions that could be analyzed were oriented at a low angle or parallel to the surface of the ventricle. These are putative examples of symmetric, self-renewing divisions since both daughters remain within the ependymal layer and inherit determinants equally.

The distribution of mitotic spindle angles suggests there are about equal numbers of symmetric and asymmetric divisions within the activated ependymal layer. However, although most prior studies of neuro-epithelial cells in the developing mammalian brain reported that that vertical divisions are neurogenic and therefore asymmetric (Chenn and McConnell, 1995) it has been reported more recently that divisions parallel to the surface may also be asymmetric (Haydar et al., 2003; Konno et al., 2008). Such low angle divisions may be asymmetric because even slight angles can result in one daughter being forced out of the ependymal layer as in Fig. 3e and result in differing inheritances by each daughter cell. It therefore seems likely that some of the low angle divisions which we counted as symmetric are actually asymmetric and that symmetric, self-renewing divisions are relatively rare in this system.

The coordinated incorporation of BrdU by ependymal cells and their SVZ neighbors demonstrates that proliferative units persist in the adult mammalian brain. The dilution of label in the SVZ after 5 days of chase along with the weaker incorporation of label by ependymal cells in the pulse at 28 days confirms that ependymal cells are slowly dividing within these proliferative units. Given that these conical groups of cells were formed after short chase periods we presume that the cells of a coordinated unit are clonally related from an earlier proliferative event. Clonally related groups of ventricular zone cells which are synchronously in the cell cycle have previously been reported in the developing mouse brain (Cai et al., 1997) and similar associations between single labeled ependymal cells and labeled putative SVZ clones have also been reported in the adult mammalian brain (Johansson et al., 1999). The prevalence of these proliferative units in the adult brains here suggests that at least some members of clonal units remain in close physical proximity long after the proliferative event which generated them and that their coordination in the cell cycle is maintained.

Lentiviral labeling of the ependymal layer with constitutively expressed GFP demonstrated that ependymal cells are clonally related to SVZ cells, and we assume that the ependymal cells are the progenitors of these clones. Our data also show that cells clonally derived from the ependymal layer can express the immature neuron marker βIII-tubulin as they migrate radially into the parenchyma. Furthermore, prior studies of this PD-model reported differentiation of neurons from cells that incorporated BrdU in response to the stimuli (Fallon et al., 2000). While it is theoretically possible that independent lentiviral infections of adjacent cells could produce the artificial appearance of clonality among adjacent cells, the corroborating BrdU evidence makes the random coincidence of an isolated group of GFP-labeled cells cycling at approximately the same time and speed extremely unlikely. In addition, the idea that some SVZ astrocytes were directly infected by the lentivirus since they sometimes contact the ventricle via an apical process (Doetsch et al., 2002; Merkle et al., 2004) is unlikely to account for many clones since the number of SVZ astrocytes that contact the ventricle (Fig. 4d) decreases sharply with age, from 22.1% at P15 to 1.4% by 4–6 weeks of age in male mice (Doetsch et al., 2002; Merkle et al., 2004) and the ependymal cells in clones reported here are located immediately adjacent to the ventricle surface where they are clearly distinguishable from SVZ astrocytes.

Based on our findings we conclude that in PD-model rats ependymal cells divide symmetrically to self-renew and asymmetrically to transfer progeny into the SVZ. Since the evidence indicates that they are neurogenic in vivo, we conclude that ependymal cells are the true NSCs. The SVZ progeny of the ependymal layer appear to function as TACs (Betschinger and Knoblisch, 2004; Wodarz and Huttner, 2003) to magnify the output of ependymal proliferation, and their progeny appear to migrate to other areas of the adult brain. Although both ependymal cells and SVZ cells may have the capacity to proliferate in response to injury, correct identification of the adult NSC is important because TACs may be more restricted in potential and therefore less effective for therapeutic applications than the true stem cells of the ependymal layer.

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