Biochemical and Biophysical Research Communications 450 (2014) 378–383

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cerebellar stem cells do not produce neurons and astrocytes in adult mouse

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ARTICLE INFO

Article history:
Received 19 May 2014
Available online 2 June 2014

Keywords:
Cell proliferation
Stem cells
Neurogenesis
Adult mouse cerebellum

ABSTRACT

Although previous studies implied that cerebellar stem cells exist in some adult mammals, little is known about whether these stem cells can produce new neurons and astrocytes. In this study by bromodeoxyuridine (BrdU) intraperitoneal (i.p.) injection, we found that there are abundant $BrdU^+$ cells in adult mouse cerebellum, and their quantity and density decreases significantly over time. We also found cell proliferation rate is diversified in different cerebellar regions. Among these $BrdU^+$ cells, very few are $mash1^+$ or $nestin^+$ stem cells, and the vast majority of cerebellar stem cells are quiescent. Data obtained by *in vivo* retrovirus injection indicate that stem cells do not produce neurons and astrocytes in adult mouse cerebellum. Instead, some cells labeled by retrovirus are $Iba1^+$ microglia. These results indicate that very few stem cells exist in adult mouse cerebellum, and none of these stem cells contribute to neurogenesis and astrogenesis under physiological condition.

1. Introduction

In mammalian brain, adult neurogenesis is thought to take place in highly restricted sites-the forebrain subventricular zone (SVZ) and the hippocampal subgranular zone (SGZ) [1–3]. Normality of specific brain functions such as learning, memory, olfaction, and mood modulation requires participation of adult-born neurons [4,5]. Recent years, the occurrence of "incomplete" neurogenic and gliogenic processes had been found in some non-neurogenic regions of adult mammalian brain, such as hypothalamus [6,7], brainstem [8], striatum [9], amygdala [10,11]. In terms of mammalian cerebellum, it has been long believed that neurogenesis is limited to early postnatal stage, and no neurogenesis exists in adult [11–13]. However, recent studies on New Zealand white rabbit showed that cerebellar neurogenesis can also be maintained into adulthood [14–17]. Moreover, a research in cat cerebellum suggested neurogenesis of unipolar brush cells (UBCs), one subtype of cerebellar interneurons, may continue for several postnatal months [18]. In contrast, other studies revealed that neurogenesis is complete by about 3–4 postnatal weeks in cat cerebellum [19,20]. Of note, a recent study found the expression of polysialylated neural cell adhesion molecule (PSA-NCAM) and doublecortin (DCX), markers for newborn and migrating immature neurons, in UBCs in adult rat cerebellum [21].

In this study, we directly inspected whether neurogenesis takes place in adult mouse cerebellum by a combination of BrdU i.p. injection, EGFP retrovirus *in vivo* delivery and immunohistochemical analysis. We identified that very few stem cells exist in adult mouse cerebellum, and none of these stem cells contribute to neurogenesis and astrogenesis under physiological condition.

2. Materials and methods

2.1. Bromodeoxyuridine labeling

All experiments and animal care were operated under the guidelines of Fudan University Shanghai Medical College. 2-month-old and 9-month-old CD-1 mice received intraperitoneal injection of bromodeoxyuridine (BrdU, 25 mg/kg body weight, Sigma) following the protocol described in Results.

Mice were intracardially perfused with cold phosphate-buffered saline (PBS, pH 7.4) and cold 4% paraformaldehyde (PFA) in PBS. The brains were fixed in 4% PFA overnight, washed in PBS, and coronally sectioned at 60 μm by vibratome (VT1000S, Leica). Sections were pretreated by 2 N HCl for one hour at 37 °C, followed

Abbreviations: BrdU, bromodeoxyuridine; i.p., intraperitoneal; ML, the molecular layer; GL+PL, the granule cell layer and Purkinje cell layer; WM, white matter; DCN, the deep cerebellar nuclei; UBCs, unipolar brush cells; PSA-NCAM, polysialylated neural cell adhesion molecule; DCX, doublecortin; EGFP, enhanced green fluorescent protein.

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http://dx.doi.org/10.1016/j.bbrc.2014.05.131
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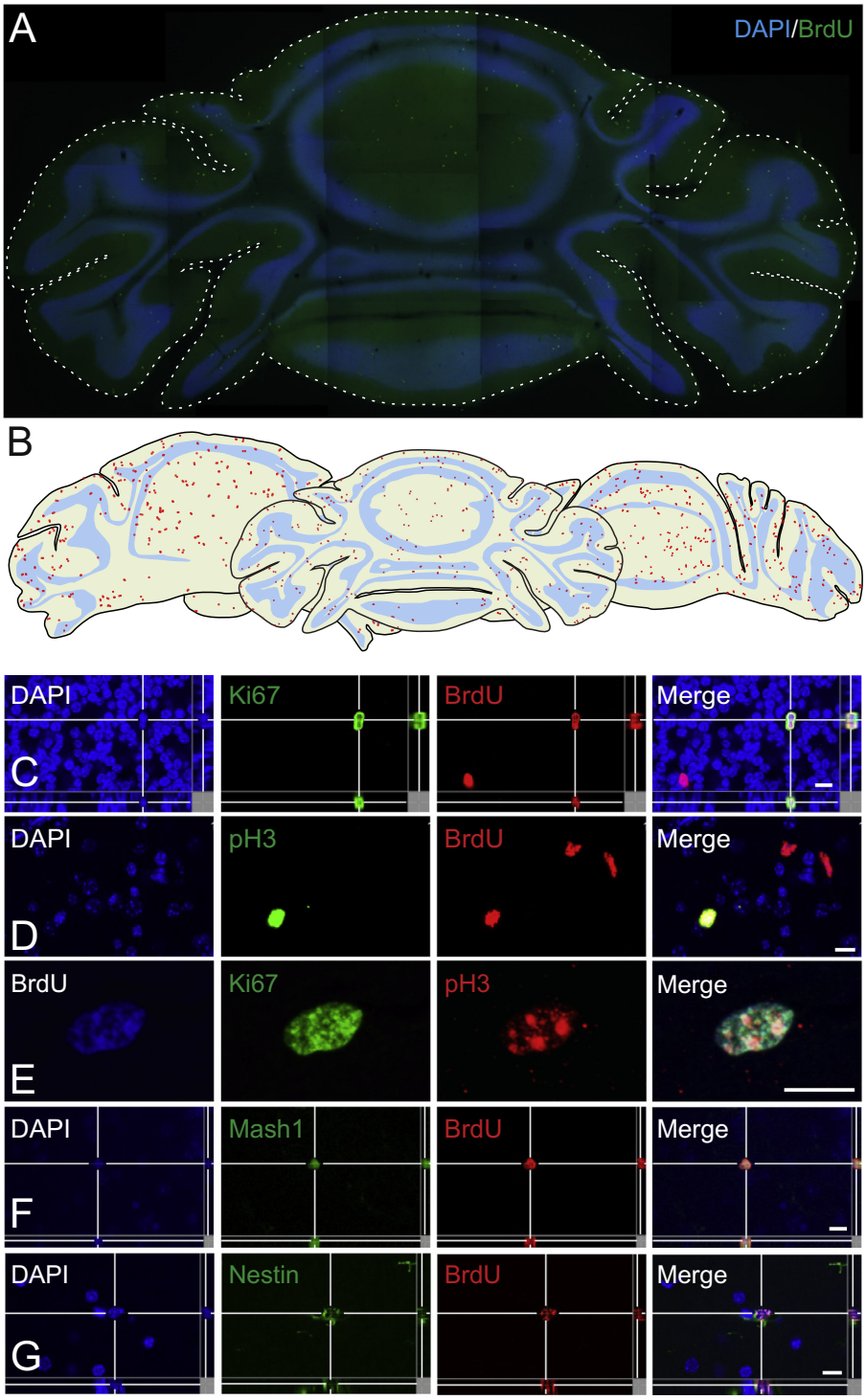

Fig. 1. Abundant BrdU$^+$ cells distributed in adult mouse cerebellum, but very few of them are stem cells. (A) Representative image of adult mouse cerebellum immunostained with antibodies against BrdU and DAPI after series of BrdU i.p. injections. (B) Plots illustrating the distribution of BrdU$^+$ cells in cerebellum. (C and D) Representative images showing colocalization of BrdU with proliferating cell markers Ki67 and pH3, respectively. (E) Tri-labeling between BrdU, Ki67 and pH3. (F and G) Representative image showing colocalization of BrdU with stem cell marker mash1 and nestin. Scale bar: 10 μm.

by 0.1 M boric acid (pH 8.44) for 30 min. Primary antibodies in donkey serum solution (10% donkey serum, 0.5% Triton-X-100) were applied for 48 h at 4 °C, and then appropriate secondary antibodies were applied for 2 h in the dark at room temperature. Sections were mounted and visualized under confocal laser scanning microscope (FV1000, Olympus) or epifluorescence microscope (BX41, Olympus) and processed by Imaris (Bitplane), Neurolucida (Microbrightfield) and Adobe Photoshop CS5 (Adobe system). The following primary antibodies were used: rat anti-BrdU (1:100, AbD), rabbit anti-phosphorylated histone H3 (pH3, 1:500, Millipore), mouse anti-Ki67 (1:500, DakoCytomation), mouse anti-mash1 (1:400, BD pharmingen), mouse anti-nestin (1:100, Developmental Studies Hybridoma Bank), mouse anti-parvabumin (PV, 1:500, Millipore), mouse anti-calbindin (CB, 1:5000, Swant), goat anti-calretinin (CR, 1:500, Millipore), mouse anti-NeuN (1:500, Millipore). The secondary antibodies: goat anti-rat (Alexa

Fluor 555, 1:250, Invitrogen), mouse anti-rat (Alexa Fluor 488, 1:250, Jackson ImmunoResearch), donkey anti-goat (Alexa Fluor 488 and Alexa Fluor 555, 1:250, Invitrogen), donkey anti-rabbit (Alexa Fluor 488 and Alexa Fluor 555, 1:250, Invitrogen), donkey anti-mouse (Alexa Fluor 488 and Alexa Fluor 555, 1:250, Invitrogen).

2.2. Retrovirus in vivo delivery

We used a replication-incompetent retrovirus in which the expression of EGFP is driven by the promoter UBC. Retrovirus was produced as described previously [22]. Titers ranged between 1 and 2 × 10^7 cfu/ml. All surgeries were performed under stereotaxic guidance (Stoelting). Mouse was deeply anaesthetized by an isoflurane–oxygen mixture. A pulled glass micropipette, which has been "backfilled" with mineral oil, attached to Nanoinject II auto-nanoliter injector (Drummond), then the glass micropipette was filled with retrovirus. We chose 2 or 3 sites randomly in each cerebellum, and each site was injected about 100 nl retrovirus at rate of 2.3 nl/s. After injection, the glass micropipette was remained for additional 10 min. 3 weeks and 5 weeks after surgery, the brains were perfused for immunohistochemical analysis.

2.3. Immunohistochemistry

Brains were perfused intracardially and sections were collected. Primary and secondary antibodies were applied as described. The following primary antibodies were used: chicken anti-GFP (1:500, Aves), mouse anti-GAD65 (1:200, Millipore), mouse anti-Iba1 (1:500, Wako), mouse anti-GFAP (1:500, Sigma), mouse anti-NeuN (1:500, Millipore). The secondary antibodies: donkey anti-mouse (Alexa Fluor 488 and Alexa Fluor 555, 1:500, Invitrogen), donkey anti-chicken (DyLight 488, 1:500, Jackson ImmunoResearch), DAPI (1:2000, Sigma). Images were taken and analyzed as described.

2.4. Statistics

All data are presented as mean ± s.e.m. All comparisons between two groups were analyzed by Student's paired *t* test, comparisons between more than two groups were analyzed by an ANOVA test. *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Abundant BrdU$^+$ cells distributed in adult mouse cerebellum, but very few of them are stem cells

In order to test whether cell proliferation takes place in adult mouse cerebellum, we administrated BrdU intraperitoneal (i.p.) injection once every 6 h for 24 h to 2-month-old adult mice. 24 h after the last injection, the brains were sectioned. Our data showed that abundant BrdU$^+$ cells distributed in adult mouse cerebellum (Fig. 1A and B). Most of BrdU$^+$ cells were observed as pairs, indicating they are daughter cells (Fig. 1A and B) [6]. In accordance with previous finding in zebrafish [23], BrdU$^+$ cells distributed

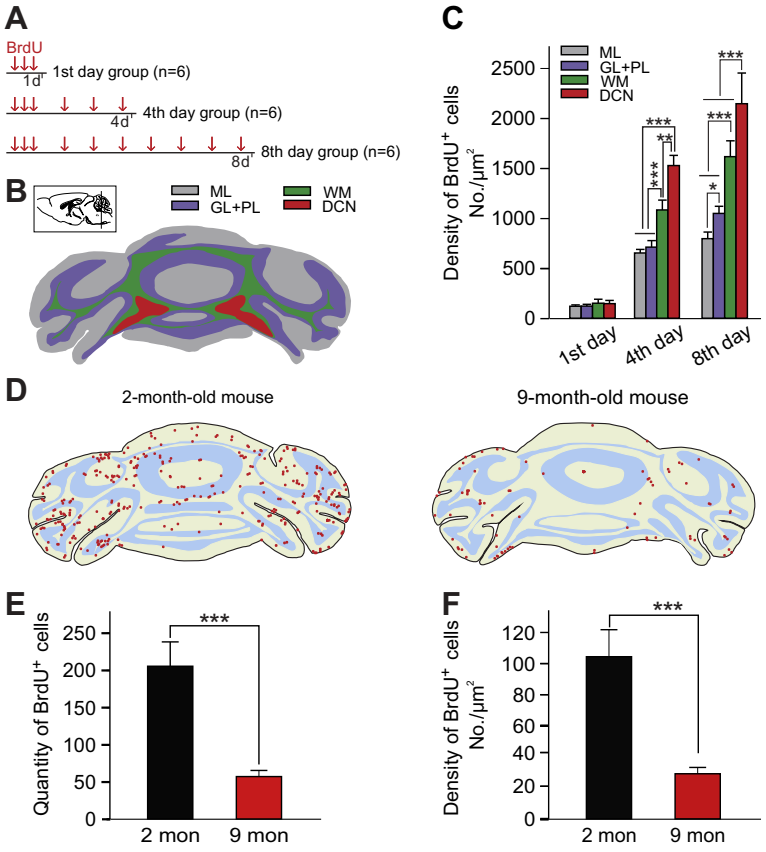

Fig. 2. The regional and age-related difference of cell proliferation in adult mouse cerebellum. (A) Experimental design to examine the cell proliferation rate in different cerebellar regions. BrdU was injected into 18 2-month-old adult mice and analyzed at the indicated time points. The sections are all from about the same Bregma level. d, day. (B) Cerebellum is roughly divided into four regions. ML, the molecular layer; GL+PL, the granule cell layer and the Purkinje cell layer; WM, white matter; DCN, the deep cerebellar nuclei. (C) Quantification of BrdU$^+$ cells in different regions within cerebellum. $n = 6$. (D) Representative plots revealing the BrdU$^+$ cells in 2- and 9-month-old mouse cerebellum. (E and F) The statistical significance of quantity and density of BrdU$^+$ cells. 2 mon, 2-month-old mice; 9 mon, 9-month-old mice. $n = 8$. $^*p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$.

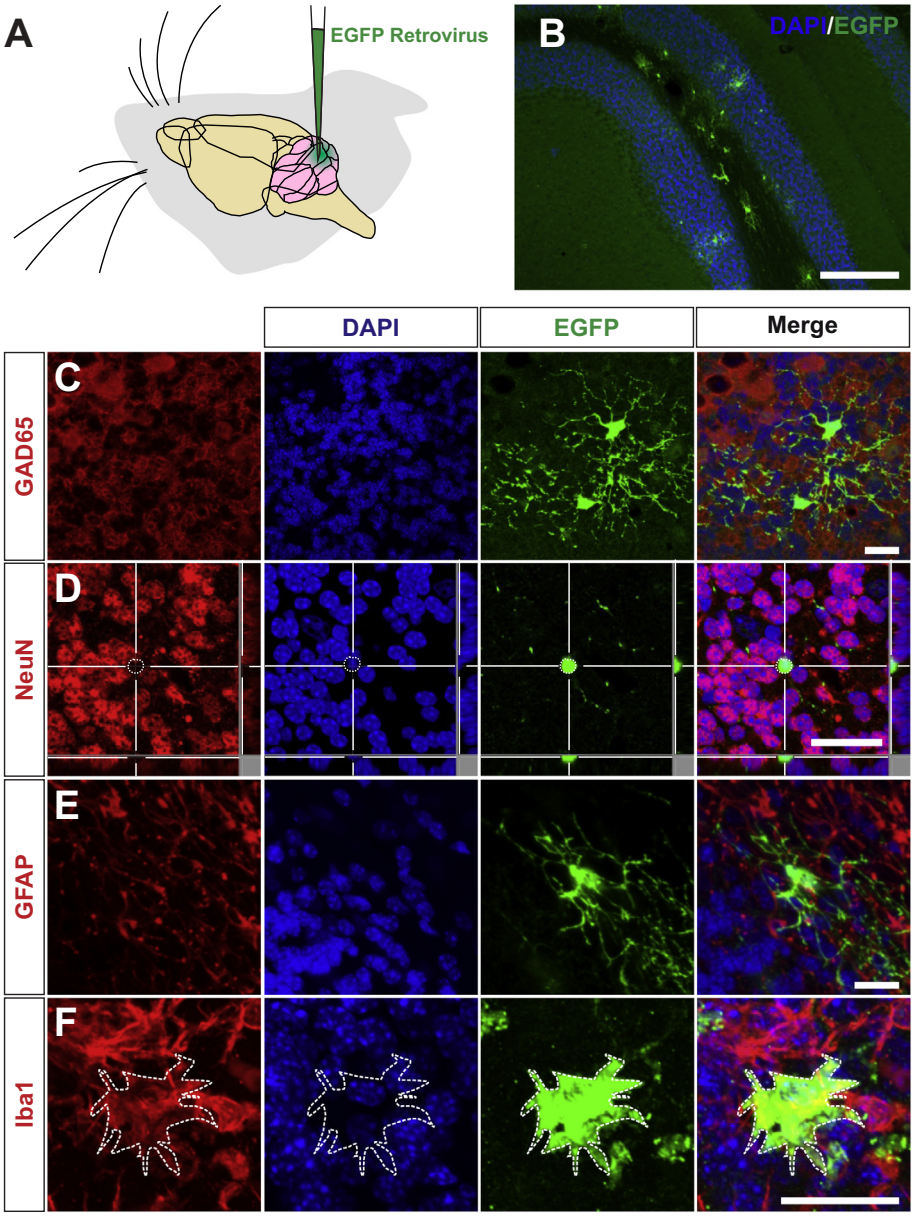

Fig. 3. No new neurons or astrocytes were generated in adult mouse cerebellum. (A) Schematic of *in vivo* retrovirus delivery. (B) Coronal section of cerebellum was immunostained with antibodies against EGFP and DAPI three weeks after retroviral injection. Scale bar: 200 μm. (C–E) $EGFP^+$ cells did not colocalize with GAD65, NeuN and GFAP, respectively. (F) Some $EGFP^+$ cells expressed Iba1. $EGFP^+$ cells were labeled by dash line in D, F. Scale bar: 25 μm.

33.4 ± 1.6% (n = 5) in the granule cell layer (GL) and the Purkinje cell layer (PL), 46.9 ± 1.7% (n = 5) in the molecular layer (ML), 11.1 ± 1.0% (n = 5) in white matter (WM), and others in the deep cerebellar nuclei (DCN). Then, we examined the colocalization of BrdU with Ki67 and phosphorylated histone H3 (pH3), markers for proliferating cells. We found some $BrdU^+$ cells expressed Ki67 or pH3 (Fig. 1C and D). Tri-labeled $BrdU^+/Ki67^+/pH3^+$ cells can also be detected (Fig. 1E).

To test whether there are proliferative stem cells in adult mouse cerebellum, we performed double immunolabeling with antibodies against BrdU and mash1. Mash1 has been identified to express in both transit amplifying and long-term neurogenic potential stem cells in the SVZ and SGZ of adult mouse brain [25]. We found only 1.3 ± 0.3% of $BrdU^+$ cells (n = 3) expressed mash1, and 12.0 ± 1.3% of $mash1^+$ cells (n = 3) expressed BrdU (Fig. 1F), indicating the vast majority of stem cells are quiescent as the same as stem cells in other adult mammalian brain regions [26,27]. In addition, colocalization of BrdU with nestin, marker for both stem cells and neural/glial progenitors, can also be occasionally detected (Fig. 1G).

These data suggest that there are abundant proliferative cells in adult mouse cerebellum, but very few of them are stem cells.

3.2. The regional and age-related difference of cell proliferation in adult mouse cerebellum

To investigate the regional difference of cell proliferation in adult mouse cerebellum, we intraperitoneally injected BrdU into adult mice with the following protocols (Fig. 2A). In the first day, we gave 18 mice (2-month-old) three BrdU i.p. injections with half an hour intervals. 6 of 18 mice were killed 1 h after the last injection (1st day group). 6 of the remaining 12 mice received one daily injection for 3 days and were killed 1 h after the last injection (4th day group). The other 6 mice received one daily injection for 7 days

and were killed 1 h after the last injection (8th day group). Then, we compared regional distribution of $BrdU^+$ cells in different groups (Fig. 2B and C). Our results showed that the order of cerebellar regions according to their cell proliferation rate from the rapidest to the slowest is DCN, WM, GL + PL, and ML (Fig. 2C). To study the effect of aging on cell proliferation in cerebellum, we gave BrdU i.p. injection to 2- and 9-month-old mice once every 6 h for 24 h, then killed the mice 24 h after the last injection. 9-month-old mice (9 mon) exhibited a significant decline in the quantity and density of $BrdU^+$ cells compared with 2-month-old controls (2 mon) (Fig. 2D–F).

3.3. No neurogenesis and astrogenesis in adult mouse cerebellum

There are two types of neurons in cerebellum based on their neurotransmitter secretion: glutamatergic granule cells and GABA (γ-aminobutyric acid)-ergic inhibitory interneurons. The major interneuron cell types in cerebellum are Purkinje cells, basket cells, stellate cells, unipolar brush cells, golgi cells and Lugaro cells [15]. In order to determine whether there are new neurons generated in adult mouse cerebellum, we also gave BrdU i.p. injection to 2-month-old mice once every 6 h for 24 h. 3 weeks later, we performed double immunolabeling with antibodies against BrdU and different makers for cerebellar neurons: neuronal nuclei (NeuN, marker for granule cells), parvalbumin (PV, marker for basket cells, stellate cells and Purkinje cells), calbindin (marker for Purkinje cells), calretinin (marker for unipolar brush cells and Lugaro cells). Our data showed that no $BrdU^+$ cells expressed these neural markers (data not shown). To provide further evidence to the question above, we chose to label proliferating cells and their progenies through retrovirus-mediated expression of enhanced green fluorescent protein (EGFP) (Fig. 3A) [22,28]. We sacrificed the mice 3 weeks after retroviral injection to leave enough time for maturation of the labeled progenies [29,30]. Some $EGFP^+$ cells can be detected in adult mouse cerebellum (Fig. 3B). We performed double immunostaining for $EGFP^+$ cells using GAD65, NeuN, and GFAP. However, none of GAD65, NeuN, and GFAP colocalized with EGFP from 3 mice (Fig. 3C–E), suggesting no interneurons, granule cells and astrocytes were generated. Instead, some $EGFP^+$ cells (29.8 ± 4.0%, from 3 mice) expressed microglia marker Iba1 (Fig. 3F). 5 weeks after retroviral injection, we also did not detect colocalization of EGFP with GAD65, NeuN, and GFAP. These data indicate that cerebellar stem cells do not produce new neurons and astrocytes in adult mouse under physiological condition.

4. Discussion

It has long believed that no neurogenesis takes place in adult mammalian cerebellum [11–13]. However, recent studies on New Zealand white rabbit have shown cerebellar neurogenesis can also be maintained into adulthood in mammals [4,24–26]. In this study, we found that stem cells do exist in adult mouse cerebellum. This result is consistent with data obtained by *in vitro* neurosphere assay, by which scientists have isolated stem cells from postnatal and adult mouse cerebellum [31,32]. In mature cerebellum, the expression of sox genes, which are treated as reliable stem cell markers, also supports our result [33,34]. Furthermore, Manohar and his colleagues reported that DCX and PSA-NCAM expressed in UBCs in adult rat cerebellum [21]. However, the recent paper published by the same lab indicated that these DCX^+ and $PSA\text{-}NCAM^+$ cells do not colocalize with BrdU, suggesting that there is no neurogenesis in adult rat cerebellum [35]. The authors suspected that DCX expression in UBCs is not limited to the developmental period but persists into, and perhaps continues for the entire duration, of adult life [35]. This result is coincident with our finding that there are no neurogenesis and astrogenesis in adult mouse cerebellum. The proliferative stem cells maybe just divide as self-renewal strategy that permits dynamical control of the population of stem cells in mature cerebellum, as described in other parts of mature brain [36–38]. *In vitro* neurosphere assay showed that postnatal cerebellar stem cells have the potential to produce granule cells, thus we suspect that *in vivo* cerebellar stem cells can generate new neurons and/or astrocytes once they get proper extrinsic and intrinsic stimulation, just like stem cells in other brain regions such as corpus callosum, thalamus, septum [11,39,40].

Since no new neurons and astrocytes are generated in adult mouse cerebellum, what are these $BrdU^+$ cells and $EGFP^+$ cells? There are several possibilities: stem cells outside the neurogenic fourth ventricle, endothelial cells and pericytes, activated microglia, or a combination of all or some of these cell types [35]. According to our result, they are at least two cell types: stem cells and microglia.

In terms of the variation of cell proliferation rate among different cerebellar regions, we do not know exactly why there are such significant variation, but it is important because it provides us new perspective to understand dynamical change of adult mammalian cerebellum. More detailed descriptions are needed to address this issue.

Acknowledgments

We wish to thank Jie Yu for assistance at various stages of this investigation, and Ji-Wen Li for BrdU labeling. This work was supported by Grants from the Natural Science Foundation of China (31200816) to Y.F., the Foundation of Ministry of Education of China (20130071110065, NCET-12-0128), Natural Science Foundation of China (31271157, 31228012), and the Foundation of Shanghai Municipal Commission of Health and Family planning (XYQ2011043) to Y.-C.Y.

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