·Article·

Changes of cell proliferation and differentiation in the developing brain of mouse

Lin QIU[1], Chang-Lian ZHU[2], Xiao-Yang WANG[2], Fa-Lin XU[2]
[1]Department of Anesthesia, [2]Department of Pediatrics, the Third Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China

Abstract: Objective To investigate the cell proliferation and differentiation in the developing brain of mouse. **Methods** C57/BL6 mice were divided into 3 groups at random. Bromodeoxyuridine (BrdU) was injected into the brains in different development periods once a day for 7 d. The brains were retrieved 4 weeks after the last BrdU injection. Immunohistochemical and immunofluorescent studies were carried out for detecting cell proliferation (BrdU) and cell differentiation (NeuN, APC, Iba1, and S100β), respectively. **Results** The number of BrdU labeled cells decreased significantly with the development of the brain. Cell proliferation was prominent in the cortex and striatum. A small portion of BrdU and NeuN double labeled cells could be detected in the cortex at the early stage of development, and in the striatum and CA of the hippocampus in all groups. The majority of BrdU labeled cells were neuroglia, and the number of neuroglia cells decreased dramatically with brain maturation. Neurogenesis is the major cytogenesis in the dentate gyrus. **Conclusion** These results demonstrated that cell proliferation, differentiation and survival were age and brain region related.
Keywords: brain development; neurogenesis; gliogenesis

1 Introduction

It is long believed that neurons are permanent cells: once damaged or dead, cannot be regenerated. However, only recently has neurogenesis become accepted as a general phenomenon in the brains of birds[1], rodents[2-5], monkeys[6], and humans[7]. Further studies demonstrated that neurogenesis persisted in the mammal forebrain throughout the whole life[8,9], and was modulated by both physiological stimuli and pathophysiological conditions[10,11]. However, there were no reports about the cell proliferation, migration, and differentiation under physiological conditions in the developing brain. In this study, 5-bromodeoxyuridine (BrdU) was used as an early marker of cell proliferation to examine the cell proliferation in the normal brain development. The phenotypes of proliferation cells were further identified by double immunofluorescence staining with antibodies against neuronal nuclear antigen (NeuN, a mature neuron marker), adenomatus polyposis coli (APC, oligodendrocyte marker), ionized calcium binding adapter molecule 1 (Iba1, an active microglia marker), or S100β (astrocyte marker).

Corresponding author: Lin QIU
Tel: 86-371-66903297
E-mail: qiulin2005@hotmail.com
Article ID: 1673-7067(2007)01-0046-07
CLC number: Q954.67
Document code: A
Received date:

2 Materials and methods

2.1 Experimental grouping and BrdU injection Eighteen C57/BL6 male mice (weighing 3–5 g) were used in the present study. The mice pups of postnatal 10 d (P10) (n = 6), 17 d (P17) (n = 6) and 24 d (P24) (n = 6), corresponding to the early stage, medium stage and late stage of the brain development, were assigned randomly into 3 groups.

BrdU is an analogue of thymidine, used as an early marker of cell proliferation. It works by substituting for thymidine during DNA replication and incorporating itself into the newly synthesized DNA during S-phase. BrdU (5 mg/mL, dissolved in 0.9% saline solution; Roche, Mannheim, Germany) was injected (50 mg/kg, i.p.) once a day for 7 d from P10, P17, or P24 in different groups. The mice pups were sacrificed 4 weeks after the last injection.

2.2 immunohistochemistry staining The animals were deeply anesthetized with 50 mg/mL phenobarbital and per-

fusion-fixed with 5% formaldehyde in 0.1 mol/L PBS for 24 h. After dehydration with graded ethanol and xylene, the brains were paraffin-embedded. The serial coronal sections were cut into 5 μm and mounted on the glass slides. The serial pair-sections with an interval of 50 sections were chosen for BrdU immunohistochemistry staining. After deparaffin and dehydration, antigen recovery was performed by boiling the sections in 10 mmol/L citrate buffer (pH 6.0) for 10 min. Nonspecific binding was blocked with 4% donkey serum in PBS for 30 min. The sections were incubated with monocolonal rat anti-BrdU primary antibody (1:100, 5 μg/mL; clone: BU1/75, Oxford Biotechnology Ltd. Oxfordshire, UK) for 60 min at room temperature, followed by biotinylated donkey anti-rat IgG (H+L) secondary antibody (1:200, 5.5 μg/mL; Jakson ImmunoResearch Lab. PA, USA) for 60 min at room temperature. Endogenous peroxidise activity was blocked with 3% H_2O_2 in PBS for 10 min. Visualization was performed by using Vectastain ABC Elite (Vector Laboratories, Burlingame, CA, USA) with 0.5 mg/mL 3,3'-diaminobenzidine (DAB) and enhanced with 15 mg/mL ammonium nickel sulphate, 2 mg/mL β-*D* glucose, 0.4 mg/mL ammonium chloride and 0.01 mg/mL β-glucose oxidase (Sigma, St Louis, MO, USA).

2.3 Immunofluorescence staining The phenotypes of newborn cells was determined by using double immunofluorescent staining. The antibodies, mouse monoclonal anti-NeuN, mouse monoclonal anti-APC, rabbit monoclonal anti-Iba1 and rabbit monoclonal anti-S100β, were used to detect mature neuron, oligodendrocyte, microglia and astrocyte, respectively. Sections deparaffin and antigen recovery was performed as above. After blocked with 4% donkey serum, the sections were incubated with rat anti-BrdU monoclonal antibody (1:100, 5 μg/mL; clone: BU1/75, Oxford Biotechnology Ltd. Oxfordshire, UK) and combined with either mouse anti-NeuN monoclonal antibody (1:200, 5 μg/mL; clone: MAB377, Chemicon, Temecula, CA, USA), or mouse anti-APC monoclonal antibody (1:100, 1 μg/mL; clone: CC-1; Calbiochem, Darmstadt, Germany), or rabbit anti-Iba1 monoclonal antibody (1:1000, 0.5 μg/mL; Wako, Osaka, Japan), or rabbit anti-S100β (1:1000; Swant, Bellinzona, Switzerland) in PBS at room temperature for 60 min. After washing, the sections were incubated with the secondary antibody Alexa Fluor 488 donkey anti-rat IgG (H+L) (1:150; Molecular Probes, USA) combined with either Alexa Fluor 555 donkey anti-mouse IgG (H + L) (1:150; Molecular Probes, USA) for BrdU/NeuN and BrdU/APC, or with Alexa Fluor 555 donkey anti-rabbit IgG (H+L) (1:150; Molecular Probes, USA) for BrdU/Iba1 and BrdU/S100β at room temperature for 60 min. After washing again, the sections were mounted by Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Immunofluorescent-stained sections were analyzed by using stereological system (Leica DM6000 M, Leica Microsystems Wetzlar GmbH, Germany) and laser-scanning confocal microscopy (Leica TCS, SP2, Leica Microsystems, Heidelberg, Germany).

2.4 Cell counting BrdU-positive cells were counted in the cortex, striatum, and the hippocampal regions: cornu ammonis (CA) and gyrus dentatus (DG). The areas were measured by stereological system[12]. Positive cells of each area were counted in each section of the brain and expressed as an average number of cells per mm^3.

2.5 Statistics All data were expressed as mean ± SD. ANOVA with Fisher's *post-hoc* test was used when more than two groups were compared. Differences were considered significant at $P < 0.05$.

3 Results

3.1 Cell proliferation in the developing brain Using BrdU as a marker for cell proliferation, newly generated survival cells were detected by immunohistochemistry staining. Figure 1 (A–I) showed the typical distributions of BrdU labeled cells in the cerebral cortex, striatum, CA and DG in three groups. Quantitative analysis of BrdU-positive cells within different age group was indicated in Fig. 1J. The number of BrdU labelled cells (the newborn cells) of each group decreased with the development of the brain (Fig. 1). The number of BrdU labelled cells in the P17-23 group decreased dramatically compared with that in the P10-16 group ($P < 0.001$) and there was no obvious change afterwards (Fig. 1A–C, J) in the cortex. The cell proliferation in the striatum, CA and DG decreased with brain maturation, similar to that in the cortex (Fig. 1D–I, J). Comparing the BrdU labelled cells in the different areas, we found that cell proliferation in the cortex markedly increased in the P10-16 group. In the striatum, DG and CA, the number of BrdU labelled cells decreased more and more. With the brain gradually maturing, the distribution of the newborn cells went to stabilization (Fig. 1J).

3.2 Neurogenesis in the developing brain NeuN is a mature-neuron specific nuclear protein. BrdU/NeuN double label immunofluorescence assay was to evaluate how many

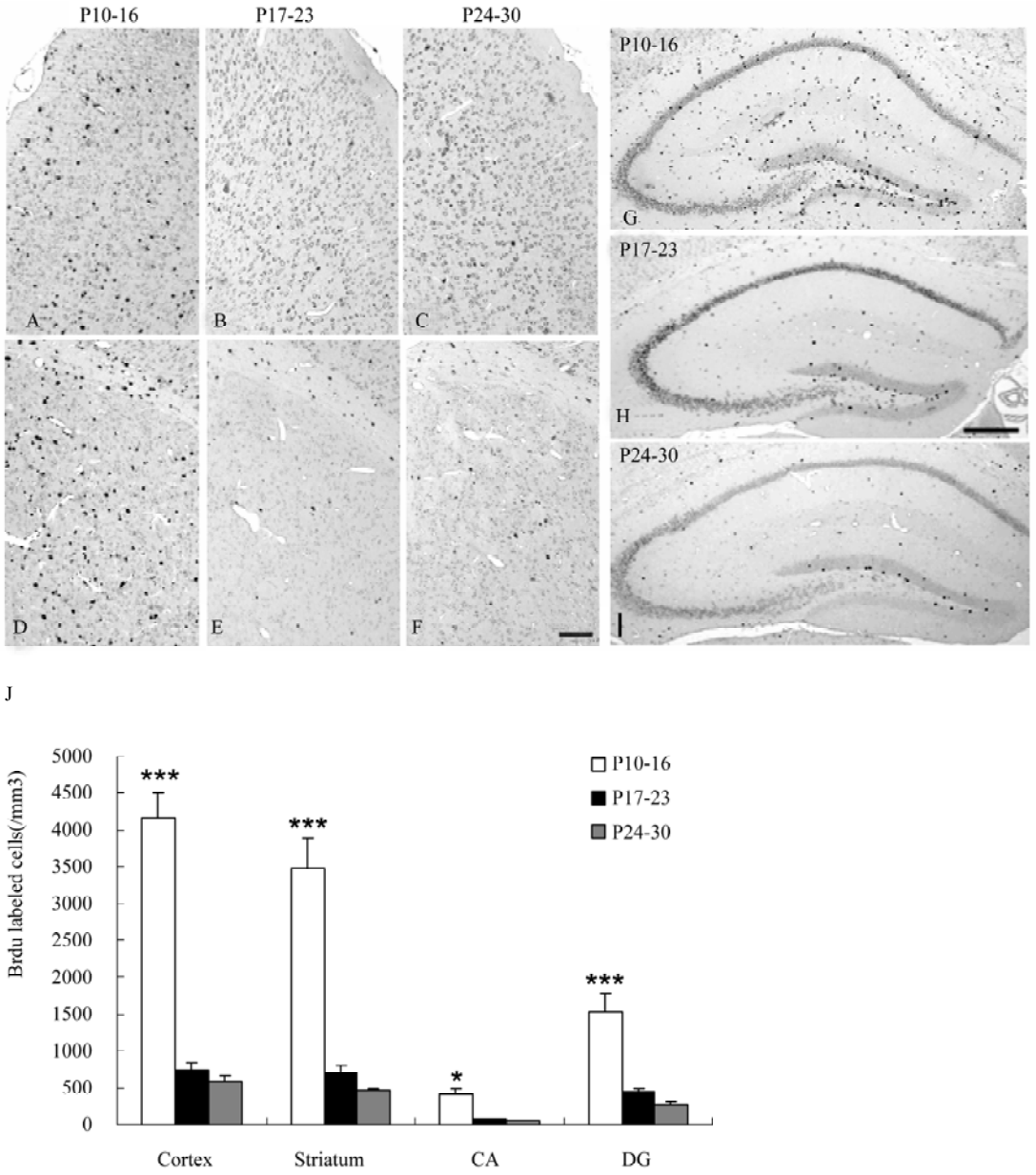

Fig. 1 Cell proliferation in different areas of the brain. Cell proliferation was detected by the BrdU labeling. A–C: the cortex, D–F: striatum, G–I: CA and DG. J: The quantification of BrdU positive cells. The number of BrdU positive cells decreased dramatically with brain development. * *P* < 0.001, * *P* < 0.05 *vs* P17-23 and P24-30. Scale bar, 150 μm in A–F, 100 μm in G–I.**

newborn cells differentiated into mature neurons.

Figure 2A showed the representative BrdU/NeuN immunofluorescence staining. Figure 3A showed quantitative analysis of neurogenesis in developing brain. In cortex, the new neurons were detected only in P10-16 group, and the cell density was (47±18) mm^3. In the striatum, the double positive cells can be detected in all groups, no significant change among them. However, the double positive cells dramatically increased in the DG in P10-16 group with the cell density of (539±145) mm^3, and decreased to (282±57) mm^3 and (161±27) mm^3 in the P17-23 group and the P24-30 group, respectively ($P < 0.001$). Comparing the neurogenesis in the different areas, we found that the new neurons in the DG were much more than that in the other areas. No significant change was observed among the other areas.

3.3 Gliogenesis in the developing brain Except for the neurogenesis, a portion of newly produced cells did not express NeuN, indicating that some cells differentiated into glial cells. In order to identify the phenotypes of the proliferating cells, double immunostaining of BrdU with APC, Iba1, or S100β were carried out.

The double labeling of BrdU and glia markers in the

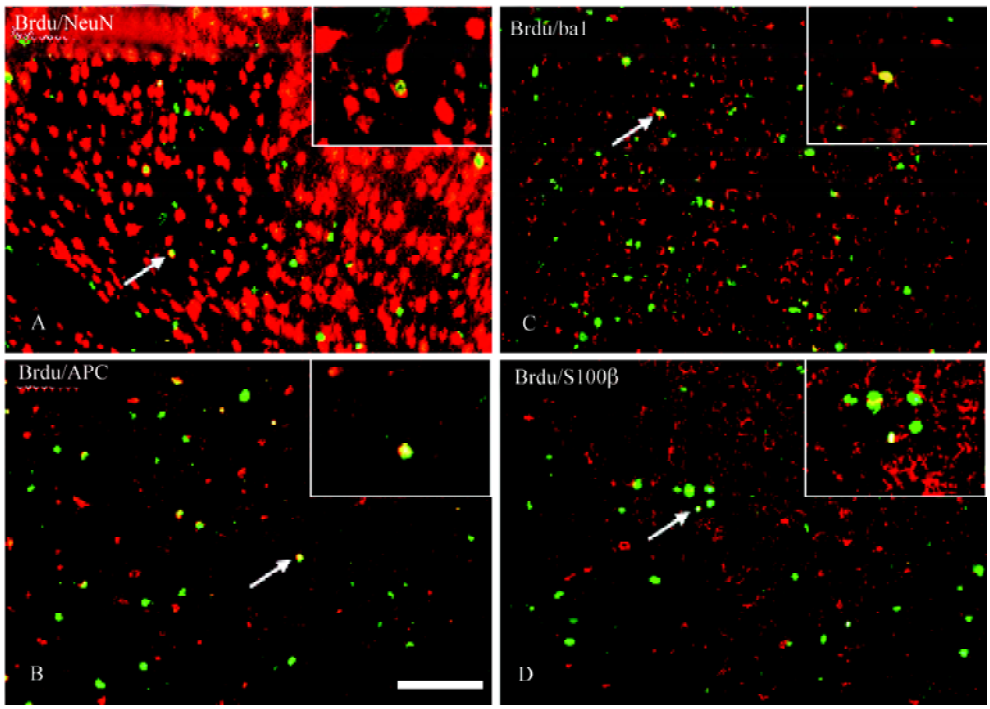

Fig. 2 Phenotype of newly generated cells. Representative BrdU (green) / NeuN (A), APC (B), Iba1 (C), S100β (D) (red) staining in cortex in P10-16 group. The arrows indicate the double positive cells (yellow). Scale bar, 100 μm.

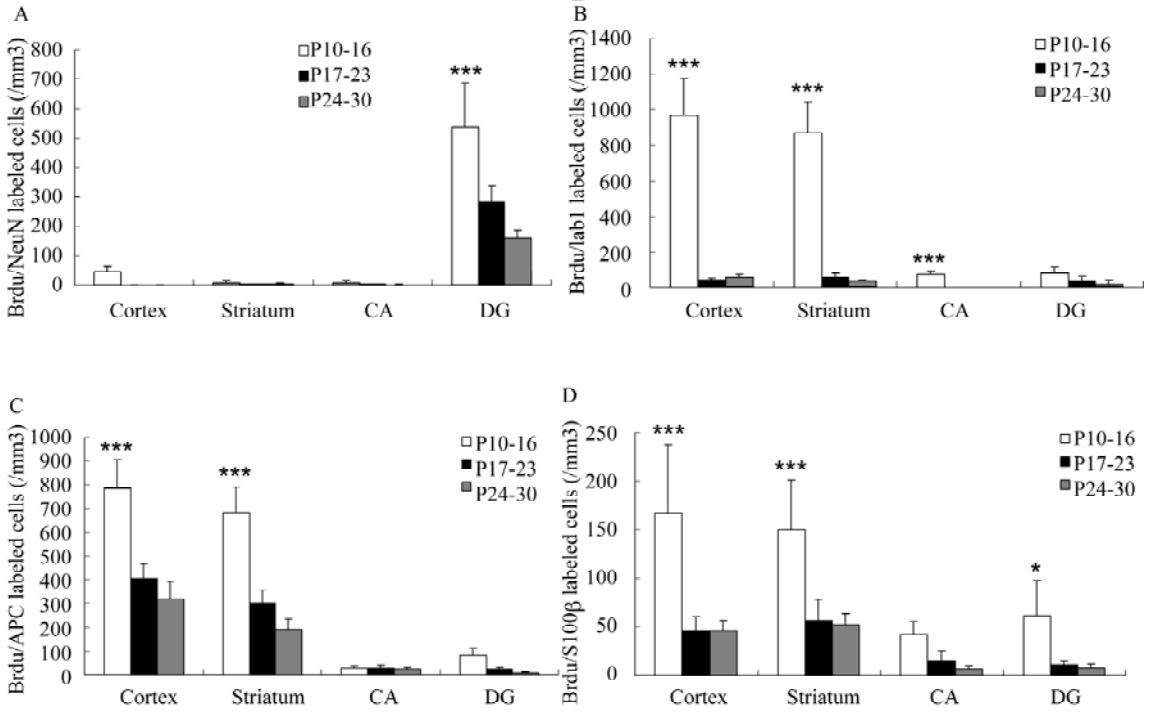

Fig. 3 Quantification of newly differentiated cells. The number of newly formed neuron and glia cells was identified by double labeling with BrdU and counted in the cortex, striatum, CA and DG under the stereology microscopy. The number of BrdU/NeuN double positive cells in the cortex and DG decreased dramatically with brain development (A). The gliogenesis decreased with brain development in each area. The number of BrdU / Iba1 (B), BrdU / APC (C) and BrdU / S100β (D) double positive cells in the cortex and striatum was the highest at the early stage of the brain development. No significant difference between the P17-23 and P24-30 group. Only BrdU/Iba1 double positive cells in the CA (B) and BrdU/S100β positive cells in the DG (D) markedly increased in P10-16 group compared with those in the P17-23 and the P24-30 group. *** $P < 0.001$, * $P < 0.05$ *vs* the P17-23 and P24-30 groups.

cortex as indicated in Fig. 2B–D. Figure 3B–D showed the quantitative analysis of gliogenesis in the developing brain. The number of the three double positive glial cells increased significantly in the cortex and striatum in the immature brain (P10-16 group). Moreover, the ratio of BrdU/APC and BrdU/Iba1 was much higher than that of BrdU/S100β, indicating that APC- (oligodendrocyte) and Iba1- (microglia) positive cells were dominant in the new generated cells. The number of these glial cells decreased significantly with brain maturation in both cortex and striatum, and the reduction was much more pronounced for the Iba1 (microglia) -positive cells (Fig. 3B–D). In the cortex, the new produced microglia in P10-16 group was 22 times higher than that in P17-23, and 16 times higher than that in P24-30. In the striatum, it was 14 times and 24 times higher than that in P17-23 and P24-30 group, respectively. The double labelling of BrdU and glia markers in the hippocampus as indicated (Fig. 3B–D). In the early term of brain development (P10-16 group), the number of BrdU/Iba1 markedly increased and declined thereafter in the CA area. However, no newly generated microglia was detected in the late stage of brain development (P24-30 group). No significant changes of BrdU/APC (oligodendrocyte) and BrdU/S100β (astrocyte) were observed among the stages of brain development. In DG, the number of BrdU labelled astrocyte decreased significantly with brain maturation ($P < 0.01$). However, there were no significant changes of microglia and oligodendrocyte during the whole period of brain development.

4 Discussion

The current study demonstrated that neurogenesis persists in the brain throughout the life, and the rate of neuronal production declined with age[8]. However, there was no report to show when the cell proliferation start to decline. This study showed that the cell proliferation, migration and survival was much higher at P10-16 and declined dramatically after that in the developing brain. Although very little is known about the exact molecular mechanisms that regulate neural stem cell proliferation, several factors have been identified to affect neurogenesis. Endogenously synthesized basic fibroblast growth factor (bFGF) stimulates neurogenesis in the subventricular zone (SVZ). The mice's genetical deficiency in bFGF exhibit a significant reduction of neurogenesis[13,14]. Heparin-binding epidermal growth factor (HB-EGF) can stimulate the neurogenesis[15]. Furthermore, the increase of brain-derived neurotrophic factor (BDNF), erythropoietin, or EGF may augment neurogenesis[16-18]. These data suggest that a variety of neurotrophic and growth factors can stimulate neurogenesis. In this study, the number of newborn cells that labelled by mature-neuron marker NeuN was higher at P10-16, corresponding to the early stage of the brain development. Some researches has demonstrated that many growth factors are expressed at high levels in the developing brain and decreased when the brain development completed[19,20]. Moreover, our study also found that in the early development stage, the cytogenesis varied in different areas of the brain. Cell proliferation was most active in the cortex and striatum, and least active in the CA area, suggesting that the proliferation of new cells is related with the microenvironment they encountered.

Neurogenesis in the cerebral cortex was discrepancy. Gould *et al*.[21] reported the genesis of new neurons in the neocortex of adult macaque monkeys. These cells arise from the SVZ and migrate through the white matter to integrate into the neocortex, where they differentiate into mature neurons. However, another study performed in macaque was not in agreement with these data and, in contrast, demonstrated that BrdU positive cells detected in the neocortex are in fact satellite glial cells closely apposed to resident neurons[22]. In the developing brain, neurogenesis has been identified in the cortex[23]. In this study, very little BrdU and NeuN double positive cells were detected in the cortex at P10-16 and no double positive cell was detected at P17-23 and P24-30. However, in striatum, CA and DG areas, the double positive cells can be detected in all the groups. Especially in DG, most newly produced cells differentiated into the neurons. Moreover, the neurogenesis was pronounced at P10-16 and decreased with the development of the brain. The age-related decline in neurogenesis is due to the rate of neuronal production declines with age and age related alteration in the cellular microenvironment slow the neuron maturation[24]. It indicates that the immature brain possess much potential capacity to generate new neurons.

Although our study demonstrated that there was neurogenesis in the cortex, striatum and CA, the total number of BrdU and NeuN double positive cells was very low. On the contrary, majority BrdU labeled cells were stained with Iba1 (microglia) or APC (oligodendrocyte). The large amount of microglia cells probably enhances neural stem cell proliferation and migration[25]. The oligodendrocyte may

indicate that myeline formation is under an active stage in the neonates[26]. It indicates that the younger the more plasticity is possessed. Astrocytes play many essential roles in normal neurotransmission[27], but astrogenesis was relatively low in our study.

In summary, the present study demonstrates that the cell proliferation, differentiation and survival were age and brain region related. Brain developmental level needs to be concerned for the future study and clinical intervention.

Acknowledgments: This work was supported by the grant of National Natural Science Foundation of China (No. 30470598).

References:

[1] Barnea A, Nottebohm F. Seasonal recruitment of hippocampal neurons in adult free-ranging black-capped chickadees. Proc Natl Acad Sci USA 1994, 91: 11217-11221.

[2] Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ. Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 1999, 2: 260-265.

[3] Kempermann G, Kuhn HG, Gage FH. More hippocampal neurons in adult mice living in an enriched environment. Nature 1997, 386: 493-495.

[4] van Praag H, van Praag H, Christie BR, Sejnowski TJ, Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci USA 1999, 96: 13427-13431.

[5] van Praag H, Kempermann G, Gage FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 1999, 2: 266-270.

[6] Gould E, Tanapat P, McEwen BS, Flugge G, Fuchs E. Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc Natl Acad Sci USA 1998, 95: 3168-3171.

[7] Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, *et al*. Neurogenesis in the adult human hippocampus. Nat Med 1998, 4: 1313-1317.

[8] Kuhn HG, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 1996, 16: 2027-2033.

[9] Sun D, Colello RJ, Daugherty WP, Kwon TH, McGinn MJ, Harvey HB, *et al*. Cell proliferation and neuronal differentiation in the dentate gyrus in juvenile and adult rats following traumatic brain injury. J Neurotrauma 2005, 22: 95-105.

[10] Enwere E, Shingo T, Gregg C, Fujikawa H, Ohta S, Weiss S. Aging results in reduced epidermal growth factor receptor signaling, diminished olfactory neurogenesis, and deficits in fine olfactory discrimination. J Neurosci 2004, 24: 8354-8365.

[11] Komitova M, Perfilieva E, Mattsson B, Eriksson PS, Johansson BB. Enriched environment after focal cortical ischemia enhances the generation of astroglia and NG2 positive polydendrocytes in adult rat neocortex. Exp Neurol 2006, 199: 113-121.

[12] Larsson E, Lindvall O, Kokaia Z. Stereological assessment of vulnerability of immunocytochemically identified striatal and hippocampal neurons after global cerebral ischemia in rats. Brain Res 2001, 913: 117-132.

[13] Yoshimura S, Takagi Y, Harada J, Teramoto T, Thomas SS, Waeber C, *et al*. FGF-2 regulation of neurogenesis in adult hippocampus after brain injury. Proc Natl Acad Sci USA 2001, 98: 5874-5879.

[14] Matsuoka N, Nozaki K, Takagi Y, Nishimura M, Hayashi J, Miyatake S, *et al*. Adenovirus-mediated gene transfer of fibroblast growth factor-2 increases BrdU-positive cells after forebrain ischemia in gerbils. Stroke 2003, 34: 1519-1525.

[15] Jin K, Mao XO, Sun Y, Xie L, Jin L, Nishi E, *et al*. Heparin-binding epidermal growth factor-like growth factor: hypoxia-inducible expression in vitro and stimulation of neurogenesis *in vitro* and *in vivo*. J Neurosci 2002, 22: 5365-5373.

[16] Craig CG, Tropepe V, Morshead CM, Reynolds BA, Weiss S, van der Kooy D. *In vivo* growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. J Neurosci 1996, 16: 2649-2658.

[17] Pencea V, Bingaman KD, Wiegand SJ, Luskin MB. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J Neurosci 2001, 21: 6706-6717.

[18] Tsai PT, Ohab JJ, Kertesz N, Groszer M, Matter C, Gao J, *et al*. A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery. J Neurosci 2006, 26: 1269-1274.

[19] Caday CG, Klagsbrun M, Fanning PJ, Mirzabegian A, Finklestein SP. Fibroblast growth factor (FGF) levels in the developing rat brain. Brain Res Dev Brain Res 1990, 52: 241-246.

[20] Plata-Salaman CR. Epidermal growth factor and the nervous system. Peptides 1991, 12: 653-663.

[21] Gould E, Reeves AJ, Graziano MS, Gross CG. Neurogenesis in the neocortex of adult primates. Science 1999, 286: 548-552.

[22] Koketsu D, Mikami A, Miyamoto Y, Hisatsune T. Nonrenewal of neurons in the cerebral neocortex of adult macaque monkeys. J Neurosci 2003, 23: 937-942.

[23] Hayashi T, Iwai M, Ikeda T, Jin G, Deguchi K, Nagotani S, *et al*. Neural precursor cells division and migration in neonatal rat brain after ischemic/hypoxic injury. Brain Res 2005, 1038: 41-49.

[24] Jin K, Sun Y, Xie L, Batteur S, Mao XO, Smelick C, *et al*. Neurogenesis and aging: FGF-2 and HB-EGF restore neurogenesis in hippocampus and subventricular zone of aged mice. Aging Cell 2003, 2: 175-183.

[25] Aarum J, Sandberg K, Haeberlein SL, Persson MA. Migration and differentiation of neural precursor cells can be directed by microglia. Proc Natl Acad Sci USA 2003, 100: 15983-15988.

[26] Zaidi AU, Bessert DA, Ong JE, Xu H, Barks JD, Silverstein FS,

et al. New oligodendrocytes are generated after neonatal hypoxic-ischemic brain injury in rodents. Glia 2004, 46: 380-390.
[27] Fields RD, Stevens-Graham B. New insights into neuron-glia communication. Science 2002, 298: 556-562.

小鼠脑组织发育期细胞增生与分化的改变

邱林[1]，朱长连[2]，王小阳[2]，徐发林[2]
郑州大学第三附属医院，[1]麻醉科，[2]儿内科, 郑州 450052

摘要：目的 探讨小鼠脑组织发育期间的细胞增生与分化。**方法** C57/BL6小鼠分别于出生后10天（P10）、17天（P17）、24天（P24）不同脑发育期，每天注射新生细胞标记物5-溴脱氧尿嘧啶核苷（BrdU），连续注射7天，并分别于末次注射后四周将小鼠处死、取脑。采用免疫组化染色及免疫荧光染色分别检测细胞增生（BrdU）与细胞分化（NeuN、APC、Iba1和S100 β）。**结果** 细胞增生随着脑组织发育快速下降，并以皮层和纹状体区细胞增生最显著。皮层在发育早期以及纹状体和海马CA区在发育各期检测到极少数为新生神经元细胞，多数分化为胶质细胞；海马齿状回以神经元细胞再生为主；胶质细胞的再生随脑组织发育的成熟而逐渐减少。**结论** 研究证实小鼠脑组织细胞的增生、分化以及存活与发育时期、脑组织区域相关。
关键词：大脑发育；神经元细胞再生；胶质细胞再生