International Journal of Oral Biology, Vol. 33, No. 2, June 31 2008, p. 59~64
Copyright ⓒ 2008, *The Korean Academy of Oral Biology*

International Journal of Oral Biology

Age-Related Changes of Adult Neural Stem Cells in the MouseHippocampal Dentate Gyrus

Ji-Yeon Jung, Kang-Ok Byun, Yeon-Jin Jeong and Won-Jae Kim*

Dental Science Research Institute, Brain Korea 21 Project, School of Dentistry Chonnam National University, Gwangju 500-757, Korea

(Received May 6, 2008 ; Revised May 28, 2008 ; Accepted May 29, 2008)

This study was designed to investigate the changes in the properties of the neuronal setm cells or progenitor cells associated with age-related decline in neurogenesis of the hippocampal dentate gyrus (DG). Active whole cells cycle marker Ki67 (a marker of whole cell cycle)-positive and S phase marker bromodeoxyuridine (BrdU)-positive. Neural stem cells gradually were reduced in the hippocampal subgranular zone (SGZ) in an age-dependant manner after birth (from P1 month to P1 year). The ratio of BrdU-positive cells/Ki67-positive cells was gradually enhanced in an age-dependent manner. The ratio of Ki67-positive cells/ accu-mulating BrdU-positive cells at 3 hrs after BrdU injection was injected once a day for consecutive 5 days gradually decreased during ageing. TUNEL- and caspase 3 (apoptotic terminal caspase)-positive cells gradually decreased in the dentate SGZ during ageing and immunohistochemical findings of glial fibrillary acid protein (GFAP) were not changed during ageing. NeuN, a marker of mature neural cells, and BrdU-double positive cells gradually decreased in an age-dependent manner but differentiating ratio and survival rate of cells were not changed at 4 wks after BrdU injection once a day for consecutive 5 days. The number of BrdU-positive cells migrated from the hippocampal SGZ into granular layer and its migration speed was gradually declined during ageing. These results suggest that the adult neurogenesis in the mouse hippocampal DG gradually decrease through reducing proliferation of neural stem cells accompanying with cells cycle change and reduced cells migration rather than changes of differentiation.

Key words: Neural Stem Cells, Hippocampal Dentate Gyrus, Cell cycle, Proliferation, Differentiation, Apoptosis

Introduction

Over the past decade it has been well established that new neurons are continuously generated in mammalian brain throughout adult life from NSCs or progenitor cells residing in both the subventricular zone (SVZ) of the lateral ventricle and the SGZ of the hippocampal DG where is important for learning and memory function (Reynold and Weiss, 1992; Gage *et al*., 1995; Lois *et al*., 1996). NSCs in the SVZ and SGZ are self renewing and multipotent cells that give rise to neurons, astrocytes, and oligodendrocytes and seem to be astrocyte-like in appearance that express vimentin and GFAP (Sanai *et al*., 2004). NSCs with their cell bodies located within the SGZ in the DG have radial processes that project into granular cell layer and short tangential processes that extend along the border between the granular cell layer and hilus (Eriksson *et al*., 1998). NSCs of the SVZ convert into neuroblast, migrate long distance tangentially through rostral migratory stream and differentiate into interneuron in olfactory bulb, whereas NSCs of the hippocampal SGZ migrate a short distance into the granular cells layer where differentiate into projection neuron (Kuhn *et al*., 1996).

Even if the roles of the newborn neuron in the hippocampal DG are not still clear, recent reports have shown that the new neuron may play pivotal roles in cognition and brain repair. For example, water maze exercise and enriched

*Corresponding author: Won-Jae Kim, Department of Oral Physiology, School of Dentistry Chonnam National University, Gwangju 500-757, Korea. Tel.: 82-62-530-4881, Fax.: 82-62-530-4885; E-mail: wjkim@chonnam.ac.kr

environment which is associated with improved memory function and synaptic plasticity enhance adult neurogenesis in the hippocampal DG (Kempermann *et al*., 1997; van Praag *et al*., 1999). In addition, adult neurogenesis increases in hippocampal DG and cortex after brain stroke, selective lesion and seizure (Parent, 1997; Lui *et al*., 1998; Magavi *et al*., 2000).

Adult neurogenesis in the hippocampal DG can be divided into 5 stages. 1) Proliferation: NSCs give rise to transient amplifying cells and proliferating progenitor cells are tightly associated with astrocytes and vascular structure. 2) Differentiation and survival: Transient amplifying cells differentiate into immature neuron. 3) Migration: Immature neurons migrate a short distance into the granular cells layer. 4) Axon/dendrite targeting: Immature neurons extend their projecting axon along mossy fiber pathway to the CA3 pyramidal cells layer and their short dendrites into the molecular layer. 5) Synaptic integration: New granular neurons receive inputs from entorhinal cortex and send outputs to the CA3 and hilus regions (Ming and Song, 2005).

Adult neurogenesis in the dentate SGZ has been reported to be affected by various factors such as genetic controls, hormones, growth factors, neurotransmitters, behaviors, stress, external environments and ageing process (Ming and Song, 2005). Ageing is an incontrovertible fact in that it reduces neurogenesis in the hippocampal DG and makes the NSCs and progenitor cells senescent (Kuhn *et al*., 1996; Maslov *et al*., 2004). Recent reports demonstrated that adrenal steroid hormones and $P16^{INK4a}$ may contribute to the ageing-associated decline of neurogenesis in the hippocampal DG (Cameron and Gould, 1994; Molofsky *et al*., 2006). However, little is precisely known about the influence of ageing on adult neural stem cells properties including cells cycle change, proliferation, differentiation, migration and cells survival after differentiation in the hippocampal DG, although proliferation of NSCs is known to be reduced in aged rat.

The propose of this study is to investigate the changes of the NSCs and the properties of progenitor cells associated with age-related decline in neurogenesis of the hippocampal DG.

Materials and Methods

Animal preparation

Male C57BL/6J mice of various ages were used. All experiments were approved by the Animal Care and Use Committee of Chonnam National University. Bromodeoxyuridine (BrdU; Sigma, St. Louis, MO) was dissolved in 0.9% NaCl, filtered sterile and injected intraperitoneally (50 mg/kg, i.p.) according to the protocols indicated below. To examine cells cycle and the number of proliferating neural stem cells in the hippocampal DG, BrdU was injected once a day and mice were sacrificed at 3 hrs after BrdU injection. To study cells survival rate and differentiation rate of neural stem cells, BrdU was injected once for consecutive 5 days and mice were sacrificed at 3 hrs and 4 wks after the final BrdU injection. Animals were perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by fresh, cold 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were dissected and postfixed overnight with 0.1 M PBS containing 4% PFA at 4°C, washed for 6 hrs in PBS at 4°C, and cryoprotected overnight in 0.1 M PBS containing 30% sucrose at 4°C. Brains were then embedded in freezing media (optimum cutting temperature), frozen in chilled isopentane (–25°C), and stored at –80°C until sectioning. Brains were cryocutted sagittally or coronally (40 m) using a cryostat (model CM3050; Leica Microsystems, Bannockburn) and stored in cryoprotectant solution (25% ethylene glycol, 25% glycerol, and 0.05 M sodium phosphate buffer, Na-PB) at –20°C.

Immunofluorescent staining

Sections were washed with Na-PB and mounted on charged slides. Sections stained for BrdU were pretreated with 2 N HCl for 30 min at 37°C and neutralized with PBS before incubation in the primary antibody. Sections were incubated for 60 min in PBS containing 5% horse serum and 0.4% Triton X-100 (PBST). Using the same buffer solution, the sections were incubated overnight at 10°C in the primary antibody. Primary antibodies generated in mice, rats, and rabbits were used at the following concentrations: rat anti-BrdU (1 : 250; Accurate Chemicals, Westbury, NY), Ki67 (NCL-Ki67p, 1 : 300; Novocastra, Westbury, NY), GFAP (MAB3402, 1 : 500; Chemicon, Temecula, CA), mouse anti-neuronal nuclear antigen (NeuN, 1 : 100; Chemicon), mouse anti-nestin (marker of stem cell, 1 : 250; Pharmingen, San Diego, CA). Samples were washed three times with PBST for 10min at room temperature and blocked in PBST containing 5% horse serum for 30 min. Samples were then incubated for 2hrs with secondary antibodies conjugated to FITC (green, Jackson Immuno-Research, West Grove, PA) or CY3 (red, Jackson Immuno -Research). Samples were washed three times with PBST, stained with 10 mg/ml 4', 6'-diamidino-2-phenylindole (DAPI) (Sigma) for 10 min and coverslipped.

Cell death analysis

TUNEL assay was performed using commercially available kit (TMR red, Roche Diagnostics, Basel) and activated caspase-3 (1 : 200, Cell Signaling, Danvers, MA)-Immunofluorescent staining was done to examine apoptosis of NSCs in the hippocampal SGZ according to the immunofluorescent staining method mentioned above. TUNEL-positive and activated caspase 3-positive cells were counted throughout the hippocampal SGZ of 6 serial sections.

Quantification and image analysis

Staining was analyzed with a Nikon (Tokyo) microscope (model Diaphot 300) and LSM 510 confocal microscope (Zeiss, Thornwood, NY) equipped with an argon/krypton laser (488 nm), two helium/neon lasers (543 and 633 nm) and a Coherent (Santa Clara, CA) Chameleon two-photon laser [used at 725 nm to image DAPI staining]. Pictures were obtained with a Spot RT Slider camera and associated software (version 3.4; Diagnostic Instruments, Sterling Heights, MI) or with the LSM software (Zeiss). Colocalizations were investigated by performing *z*-stack acquisitions and three-dimensional reconstructions. Adobe Photoshop version 7.0.1 (Adobe Systems, San Jose, CA) was used to adjust contrast and brightness.

Statistics

Statistics were performed using the unpaired Student's t-test. Results are considered significant when the p value is < 0.05.

Results

Ageing gives rise to decline in number of BrdU or Ki67-positive cells and cell cycle changes in the hippocampal DG during ageing

To determine cell cycle change and how much changes is in number of proliferating cells in the hippocampal DG during ageing process, BrdU, a marker of S-phase cell cycle and Ki67, a marker of whole cell cycle that is expressed by cells in all phases of cell cycle except for early G1, were double-stained Immunofluorescent at 3 hrs after single BrdU injection (50 mg/kg i.p.). Ki67- and BrdU-labeled cells with irregularly shaped nuclei were observed in the hippocampal SGZ and hilus and BrdU-positive cells were always overlaped in Ki67-positive cells (Fig. 1). Both Ki67 and BrdU-positive cells were drastically reduced in the hippocampal DG during ageing, especially after postnatal 3 months and the ratio of BrdU-positive cells/Ki67-positive cells increased from postnatal at 4 wks to 1 year in an age-dependent manner (Fig. 2).

Ageing reduces proliferation of neural stem cell and death in the hippocampal SGZ

The adult hippocampal SGZ is known to have two stem cells population: the transient fast proliferating cells called as the type-2 cells and the quiescent slow proliferating cells called as the type-1 cells. BrdU (50 mg/kg, i.p.) was injected once a day for consecutive 5 days in order to collect both fast and slow proliferating neural stem cells in the hippocampal SGZ. To examine proliferating rate of neural stem cells in the SGZ during ageing, the ratio of Ki67-positive cells/accumulating BrdU-positive cells after consecutive 5 days-BrdU injection was calculated. This ratio for 5 days was lower in postnatal 6 month (P6M) mice than in postnatal 1 month (P1M) mice (Fig. 3).

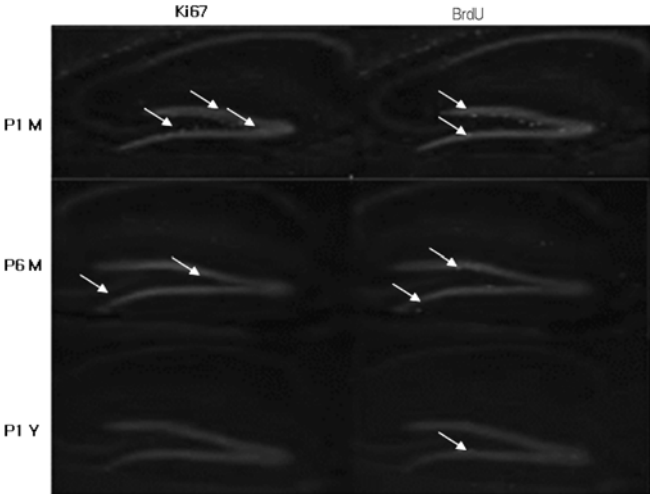

Fig. 1. Representative microphotograph of Ki67-labeled and BrdU-labeled cells in the SGZ of the hippocampal dendate gyrus at 3 hrs after BrdU injection (50 mg/kg, i.p.). postnatal month 1(P1M), postnatal year 1(P1Y).

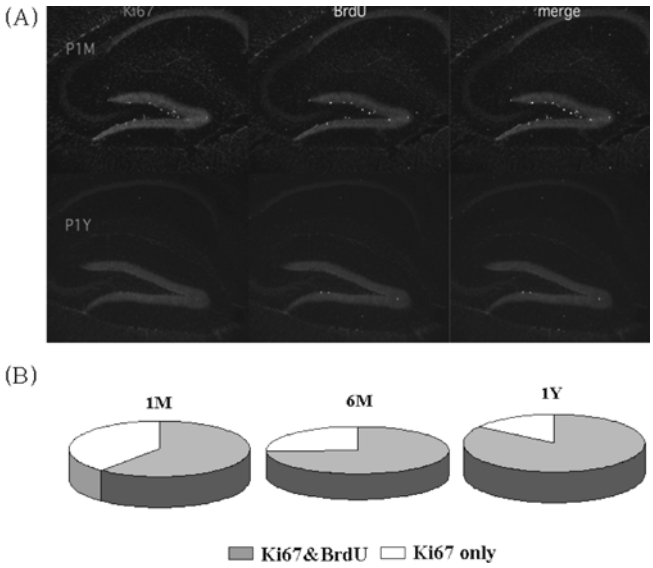

Fig. 2. Double immunofluorescent staining of Ki67-positive and BrdU-positive cells in the hippocampal SGZ at 3 hrs after BrdU injection (50 mg/kg, i.p.) (A). BrdU-positive cells/Ki67-positive cells were higher in 6 months than in 1 month. Diagram of the percentage of BrdU-positive cells/Ki67-positive cells in the hippocampal SGZ (B).

TUNEL-staining and activated caspase 3 (an apototic executing caspase)/nestin (a marker of stem cell) double immunostaining were done to investigate apoptosis of neural stem cells in the hippocampal SGZ. TUNEL-positive cells were reduced in an age-dependent manner in the SGZ and activated caspase-3/nestin-double positive cells were also diminished during aging in the SGZ (Fig. 4 and 5).

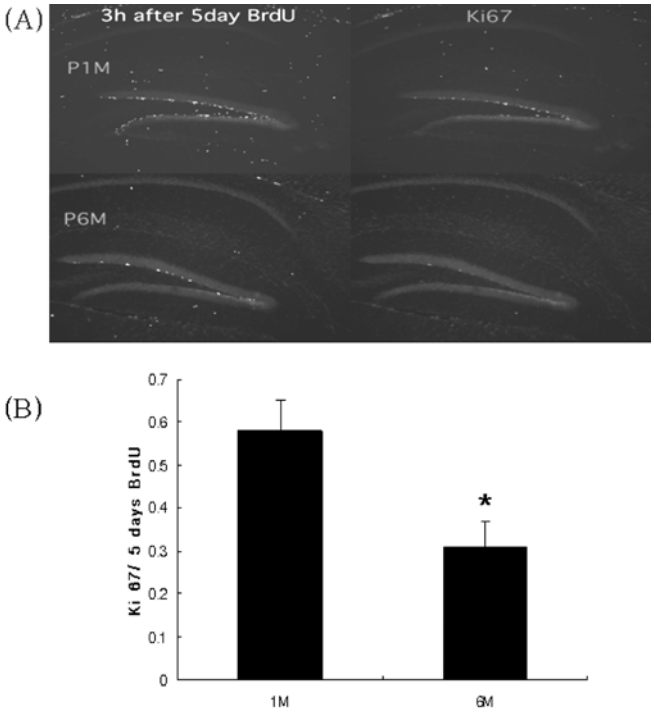

Fig. 3. Double Immunofluorescent staining of Ki67-positive and BrdU-positive cells in the hippocampal SGZ at 3 hrs after BrdU injection after consecutive 5 days (A). Histogram of the ratio of Ki67-positive cells/ accumulating BrdU-positive cells for 5 days (B). *p < 0.05, compared with 1M.

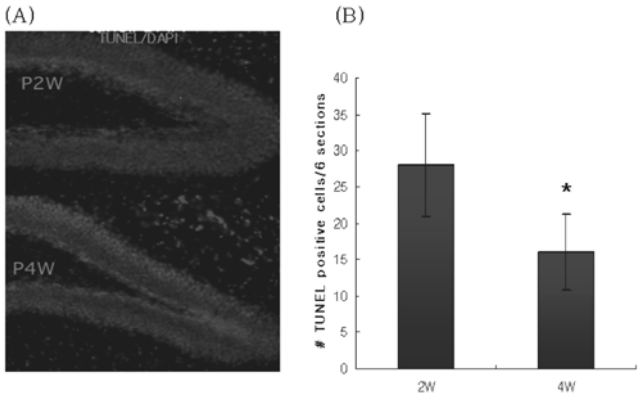

Fig. 4. TUNEL-staining in the hippocampal dentate gyrus (A) and histogram of TUNEL-positive cells from 6 serial sections (B). *p < 0.05, compared with 2W.

Neurogenesis is reduced but cell survival rate and differentiation are not altered in the hippocampal DG during ageing

To determine whether the survival rate of newborn neuron and differentiation rate of NSCs in the SGZ are changed during ageing, the number of survival BrdU-positive cells were calculated and BrdU/NeuN double-positive cells were estimated using confocal microscope at 3 hrs and 4 wks after BrdU was injected once a day for consecutive 5 days.

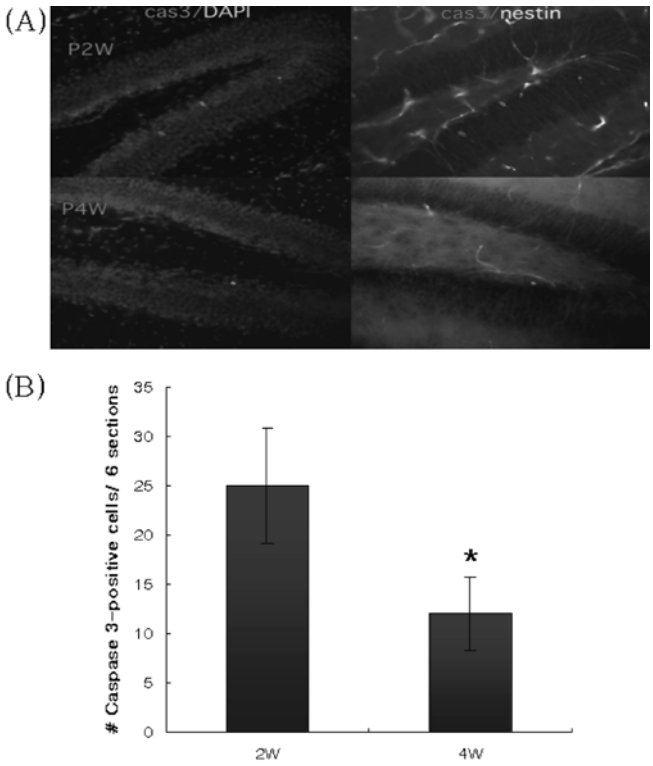

Fig. 5. Representative microphotograph of activated caspase-3/nestin double-positive cells (A) in the SGZ from 6 sections and histogram of active caspase 3/nestin double positive cells (B). *p < 0.05, compared with 2W.

Four wks after consecutive 5 days-BrdU injection, survival rate of immature neuron (BrdU-positive neurons at 3 hrs after 5 days injection/BrdU-positive cells at 4 wks after 5 days injection) was not altered in the hippocampal DG during ageing (Fig. 6).

Besides, percentage of NeuN/BrdU double positive cells of NSCs (differentiation rate) in the hippocampal DG was also not altered during ageing, although total number of BrdU/NeuN double-positive cells and total cell number migrated from SGZ into granular layer were remarkably lower in P6M than in P1M (Fig. 7).

Discussion

Adult neurogenesis continues throughout adulthood in the hippocampal DG, in order to involve in cognition or repair of lost neurons derived from injury and disease (Kempermann *et al*., 1997; Parent, 1997; Lui *et al*., 1998; Magavi, 2000). Besides, it is well documented that adult neurogenesis is reduced in the hippocampal DG and consistent with this, ageing tissues exhibit a reduced rescue capacity and an enhanced incidence of degenerative disease (Kuhn *et al*., 1996; Campisi *et al*., 2005, Lombard *et al*., 2005). Even in the present study, BrdU/Neu N double-positive cells in

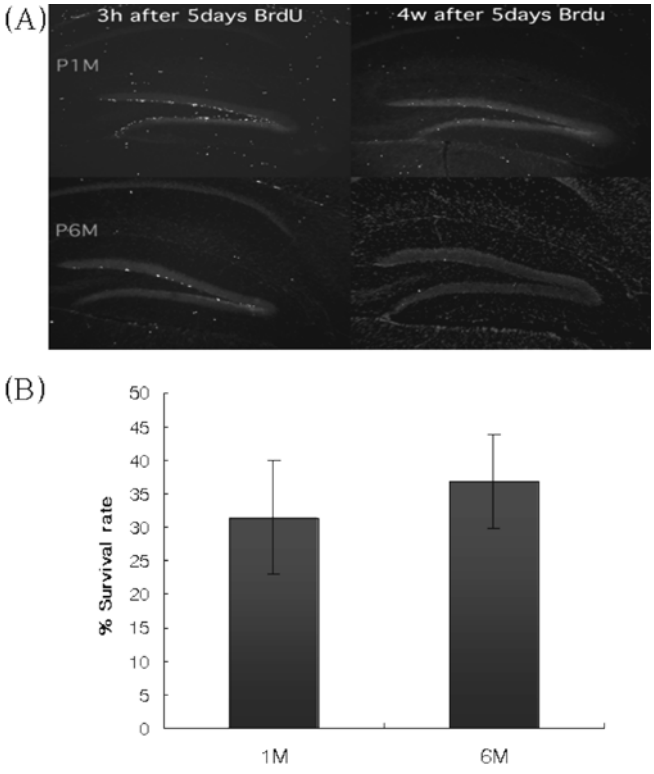

Fig. 6. Comparison of BrdU-positive cells in the hippocampal DG at 3 hrs after BrdU injection after consecutive 5 days and at 4 wks after BrdU injection after consecutive 5 days.

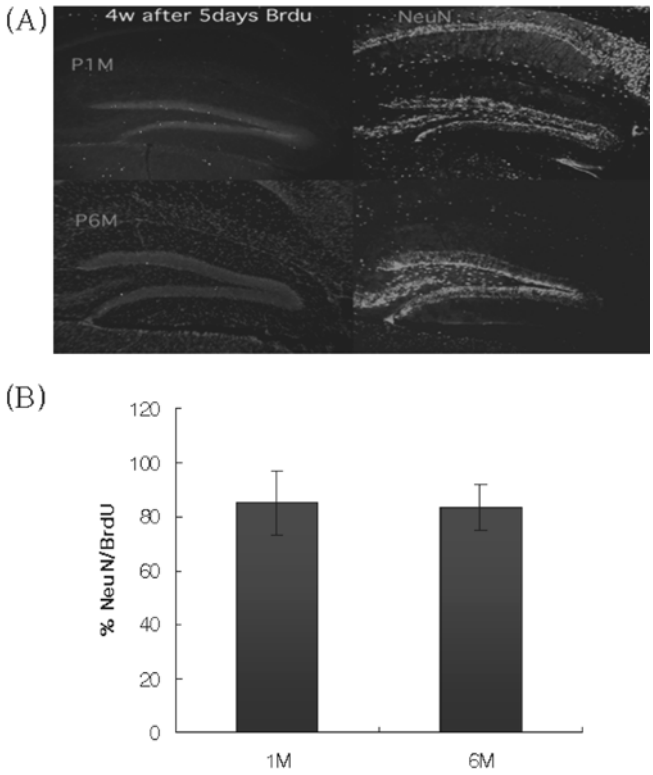

Fig. 7. Representative microphotograph of BrdU/NeuN double-positive cells in the hippocampal DG at 4 wks after BrdU injection after consecutive 5 days (A). Histogram of the ratio of NeuN/BrdU double positive cells was declined during ageing but differentiation rate was not altered (B).

the DG were remarkably reduced at 4 wks after consecutive 5 days injection of BrdU in 6 months mice than at 1 month mice, suggesting that ageing induces the decline of adult neurogenesis in the hippocampal DG, in agreement with the previous reports in rats (Kuhn *et al*., 1996). However, the ageing changes of adult stem cells properties and the mechanism responsible for the age-associated decline in adult neurogenesis have been not fully elucidated, although there is a possibility that adrenal steroid hormones and p16INK4a may contribute to the ageing-related decline of neurogenesis in the hippocampal DG (Cameron and Gould, 1994; Molofsky *et al*., 2006).

In general, adult neurogenesis can be grossly divided into 3 separated events: stem cells proliferation, cell survival and neural differentiation. In this study, numbers of Ki67-positive cells or BrdU were gradually declined in the hippocampal SGZ in an age-dependent manner, indicating that proliferating stem cells are gradually reduced in the hippocampal DG during ageing. It is speculated that there are several possibilities responsible for age-related decline in Ki67-positive or BrdU-positive cells in the hippocampal SGZ: 1) decrease in proliferating rate of stem cells without change of stem cells population, 2) reduced proliferating rate with decreased number of stem cells population, 3) decreased number of stem cells population without change of proliferating rate. It has been known that the adult hippocampal SGZ has two stem cells population: the transient fast proliferating cells called as the type-2 cells and the quiescent slow proliferating cells called as the type-1 cells (Seri *et al*., 2001; 2002). Therefore, BrdU was injected once a day for consecutive 5 days in order to collect both the fast and slow proliferating stem cells. After that, the ratio of Ki67-positive cells/5 days-accumulating BrdU-positive cells was calculated to examine the proliferating rate of neural stem cells in the SGZ. In the present study, Ki67-positive cells/accumulating BrdU-positive cells for 5 days was lower in 6 months mice than in 1 month mice, meaning that ageing could reduce proliferation rate of the SGZ neural stem cells in mouse in agreement with the previous report in rats (Kuhn *et al*., 1996).

To check another possibility whether neural stem cells death may be involved in the age-related decline in Ki67-positive or BrdU-positive cells, TUNEL staining and activated caspase-3/nestin double-immunostaining were performed. TUNEL-positive and activated caspase 3/nestin-double positive cells were fewer in 1 month mice than in 2 wks mice, indicating that apoptosis of neural stem cells can paradoxically decrease during ageing. Besides, Immunofluorescence appearances of GFAP, a marker of neural stem cell, seem to be almost same. Taken together, it is suggested that age-related decline in proliferating stem

cells of the SGZ must be derived from reduced proliferation of the neural stem cells rather than from cell death of the neural stem cells. Moreover, BrdU-positive cells/Ki67-positive cells increased in an age-dependent manner in the SGZ in this study, suggesting that S phase of the adult neural stem cells become longer during ageing, resulting into enhanced cell cycle duration. This finding shows the first evidence that S phase of proliferating adult stem cells can become longer during ageing, although cell cycle duration and S phase of neonatal neural stem cells are known to become longer during developing. From this result, it is supposed that age-associated decline in proliferation of adult neural stem cells is due to increased duration with S phase of cell cycle.

To test the survival rate of cells which can influence on adult neurogenesis, the ratio of BrdU-positive cells at 4 wks after BrdU injection for consecutive 5 days/BrdU-positive cells at 3 hrs after BrdU injection for consecutive 5 days was calculated. Cell survival rate was almost similar between 1 month mice and 6 months mice, meaning that cell survival rate is not altered during ageing. In addition, % NeuN/BrdU-double positive cells was same between in 1 month mice and 6 months mice at 4 wks after consecutive 5 days of BrdU injection, suggesting that differentiation ratio was not altered during ageing. However, number of NeuN/BrdU-double positive cells migrated from the SGZ into the granular layer and migrating speed were higher in 1 month mice than in 6 months mice, demonstrating that newborn neurons are less added from the hippocampal SGZ into the granular layer in old mouse than in young mouse.

From these results, it is concluded that age-associated decline of adult neurogenesis may be derived from decreased proliferation of adult neural stem cell with long S phase cell cycle and reduced cells migration rather than from altered cell survival rate or differentiation rate.

Acknowledgement

This work was supported by a grant from KRF(2007-0848), Chonnam national university(2007) and Chonnam national hospital(2007).

References

Cameron H. A. and Gould E.: Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. Neuroscience **61**:203-209, 1994.

Campisi J.: Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell **120**: 513-522, 2005.

Eriksson P. S., Perfilieva E., Bjork-Eriksson T., Alborn A. M. and Nordborg C.: Neurogenesis in the adult human hippocampus. Nat. Med. **4**: 1313-1317, 1998.

Gage F. H., Coates P. W., Palmer T. D., Kuhn H. G., Fisher L. J., Suhoncn J. O., Peterson D. A., Suhr S. T. and Ray J.: Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc. Natl. Acad. Sci. USA **92**: 11879-11883, 1995.

Georg K. H., Dickinson-Anson H. and Gage F. H.: Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. J. Neurosci. **76**: 2027-2033, 1996.

Kempermann G., Kuhn H. G. and Gage F. H.: More hippocampal neurons in adult mice living in an enriched environment. Nature **386**: 493-495, 1997.

Kuhn H. G., Dickinson-Anson H. and Gage F. H.: Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J. Neurosci. **16**: 2027-2033, 1996.

Lombard D. B.: DNA repair, genome stability, and aging. Cell **120**: 497-512, 2005.

Lois C., Garcia-Verdugo J.M. and Alvarez-Buylla A.: Chain migration of neuronal precursors. Science **271**: 978-981, 1996.

Magavi S. S., Leavitt B. R. and Macklis J. D.: Induction of neurogenesis in the neocortex of adult mice. Nature **405**: 951-955, 2000.

Maslov A. Y., Barone T. A., Plunkett R. J. and Pruitt S. C.: Neural stem cell detection, characterization, and age-related changes in the subventricular zone of mice. J. Neurosci. **24**: 1726 -1733, 2004.

Ming G. I. and Song H. J.: Adult neurogenesis in the mammalian central nervous system. Annu. Rev. Neurosci. **28**: 223-250, 2005.

Molofsky A. V.. Slutsky S.G., Joseph N. M., He S., Pardal R., Krishnamurthy J., Sharpless N. E. and Morrison S.J.: Increasing p16INK4a Expression decreases forebrain progenitors and neurogenesis during ageing. Nature **433**: 448-452, 2006.

Parent J. M.: Dentate granule neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult hippocampus. J. Neurosci. 17: 3727-3738, 1997.

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

Sanai N., Tramontin A. D., Quinones-Hinojosa A., Barbaro N. M. and Gupta N.: Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature **427**: 740-744, 2004.

Seri B., Garcia-Verdugo J. M., McEwen B. S. and Alvarez-Buylla A.: Astrocytes give rise to new neurons in the adult mammalian hippocampus. J. Neurosci. **21**: 7153-7160, 2001.

van Praag H., Kempermann G. and Gage F. H.: Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nature Neurosci. **2**: 266-270, 1999.