Experimental Neurobiology
Vol. 13, pages 91~96, December 2004

Proliferation of Neural Stem Cells Upregulated by Dietary Restriction in the Adult Rat Hippocampus

Hwon Heo, Taewoo Kim, Eunsil Cho,
and Yunhee Kim Kwon*

Department of Biology and Research Institute for Basic Sciences,
College of Sciences, Kyunghee University, Seoul 130-701, Korea

ABSTRACT

Neural stem cells (NSC) are multipotential progenitor cells that have self-renewal activities and believed as a source for treatment of degenerative brain disease such as Parkinson's disease and Alzheimer's disease. In this study, we investigated the proliferation and migration of neural stem cells and effect of dietry restriction to neurogenesis in the adult rat hippocampus. We observed the dividing cells in hippocampal alveus as well as in dentate gyrus by incorporation of BrdU. BrdU-labelled cells were migrated from hippocampal alveus near CA1 subfield toward CA3 in hippocampus and from subgranular cell layer to granular cell layer, following the migration pathway which neural stem cells migrate during embryogenesis. In dietry restriction (DR) models, BrdU-labelled cells were increased significantly in dentate gyrus and CA1 subfield. The present study suggestes that short-term DR improves proliferation of neural stem cells in the adult rats.

Key words: Dietary restrichion, neural stem cell, hippocampus

INTRODUCTION

Neural stem cells (NSC) are multipotential progenitor cells that have self-renewal activities. A single NSC is capable of generating various kinds of cells within the central nervous system, including neurons, astrocytes, and oligodendrocytes. The vast majority of cells in the nervous system are born during the embryonic and early postnatal period, but new neurons are continuously added in certain regions of the adult mammalian brain (Altman and Das, 1965). These neurons are thought to derive from a population of stem cells. It was shown by Reynold and Weiss (1992) that neural stem cells taken from the adult brain can be propagated in vitro. These cells had the capacity of differentiation into three cell types of the central nervous system (Reynolds and Weiss, 1996). The newly generated cells may have a function in cognition and brain repair.

Dentate gyrus and the subventricular zone (SVZ) of several species have been shown to generate new neurons in the postnatal and adult period. Granule neurons are generated throughout life from

*To whom correspondence should be addressed.
TEL: 82-2-961-0844, 0605, Fax: 82-2-966-4497
e-mail: kimyh@khu.ac.kr

a population of continuously dividing progenitor cells residing in the subventricular zone of the dentate gyrus in the rodent brain. In these two areas, there appears to be a continuous turnover of interneurons and granule cells, implying that newborn neurons replace the dying cells and, indeed, recent evidence suggests that newly generated neurons form functional synapses (Van Praag et al., 2002).

Dietary restriction (DR) can increase life span in a wide variety of species, and can reduce neuronal damage. It has been reported to improve behavioral outcome in experimental animal models relevant to the pathogenesis of several age-related neurological disorders (AJ. Bruce-Keller et al., 1999; Duan and Mattson, 1999; Yu and Mattson, 1999; Duan et al., 2001). DR may promote neuronal survival by stimulating the expression of genes that encode cytoprotective proteins such as heat-shock proteins (Duan and Mattson, 1999; Yu and Mattson, 1999) and neurotrophic factors (Duan et al., 2001). Adult rats maintained on DR condition exhibit increased resistance of hippocampal neurons to excitoxic injury and increased resistance of striatal neurons to metabolic insults (AJ. Bruce-Keller et al., 1999). More recently, in vivo, DR in adult mice increased resistance of nitro-striatal dopaminergic neuron in a Parkinson's disease model (Duan and Mattson 1999). In Alzheimer's disease patients, DR also increases resistance of neuron to the degenerative process, and vulnerability of hippocampal neurons to excitotoxity in knockin mice that express the AD-linked M146v PS1 mutation (Zhu et al., 1999; Dubey et al., 1996). These suggest that DR may improve neuronal cell survival by increasing stem cell division.

In the present study, we investigated the development of neural stem cells in the adult rat hippocampus and effect of DR on proliferation of neural stem cells by using bromodeoxyuridine (BrdU) incorporation into replicating DNA.

MATERIALS AND METHODS

Animals and BrdU administration

Adult (300～370 g) Spraue-Dawley rats were used in all experiments. All the experiments were performed in compliance with relevant laws and institutional guidelines.

To investigate the proliferation and migration of neural stem cells, rats were divided into 2 groups and BrdU was injected once a day for 2 d or 5 d. In each group, rats were sacrificed one d after and one week after injection of BrdU (50 mg/kg body weight).

In the experiment of DR, *ad libitum* (AL) rats and DR rats were divided into each 2 groups. AL groups had continual access to food and water for 4 d or 7 d, and DR groups were maintained on an every day fasting regimen with supply of water for 4 d or 7 d. Intraperitoneal injections of BrdU were carried once a day for 3 d before sacrifice.

Immunohistochemical staining

Rats were perfused transcardinally with 4% paraformaldehyde and the brains were removed, postfixed at 4°C for 4 h, and then incubated in 30% sucrose solution until they sink. The brains were frozen with OCT compound then cryo-sectioned in coronal direction at 35μm on a cryostat (MICRON).

Free-floating sections were permeablized with 0.5% Triton X-100 for 20 min and their DNA was denatured in 50% formaldehyde and 2X SSC for 2 h at 65°C, then neutralized with 0.1M sodium borate (pH 8.5). The sections were incubated in 1% BSA in PBS/10% donky serum for 15 min, then incubated with primary anti-BrdU antibody (Becton Dickinson, 1 : 500) in PBS with 1% BSA overnight at 4°C. Sections were further processed using CyIII or FITC labeled secondary goat anti-mouse IgG antibody (Jackson Lab) in 1%BSA (1 : 500). After washing with PBS, sections were mounted with Dako mounting solution and immunostained sections were scanned with a confocal laser microscope (Carl Zeiss, LSM510). To counterstain nuclei, sections were incubated with propidium iodide (PI, 6.68μg/ml) for 30 min at 4°C in shaking incubator (Adamchik et al., 2000).

BrdU-labeled cell counting and correction of statistical data

Confocal images were taken in dentate gyrus and CA1 subfield of hippocampus in each slice and proliferating nuclei were counted. Only round shape nuclei double-stained with BrdU and PI were considered as proliferating nuclei.

Counted data were tested with Mann-Whitney test (SPSS). These numbers of data were than cor-

rected for the systematic error generated by double counting of sectioned cells using the formula: P=(A){M/(L+M)} where P is the corrected number of cells per section, A is the uncorrected number of cells per section, M is the section thickness, and L is the average diameter of a cell in a section (Renfranz et al., 1991).

RESULT

Proliferation and migration of neural stem cells in the hippocampal alveus and dentate gyrus.

We investigated first cell division in the rat hippocampus to study development of neural stem cells. Adult rats were injected with nucleotide analogue, BrdU incorporated into replicating DNA once a d for 2 d or 5 d, and then brain slices were immunostained with anti-BrdU antibody and visualized with CyIII or FITC under Confocal Laser Microscope. Many BrdU-labelled cells were observed to align along subgranular zone of dentate gyrus one d after BrdU injection for 2 d (Fig. 1, 1d DG). The fewer BrdU-labelled cells were distributed in the hippocampal alveus near CA1 subfield. After BrdU injection for 5 d, there was no significant difference but more BrdU-labelled cells were observed as scattered and they formed a few cluster in dentate gyrus and hippocampal alveus (data not shown). One week after, BrdU-labelled cells were observed as scattered in the subgranular zone and some cells were found to migrate into the granular layer. In the hippocampal alveus, cells were scattered near CA1 subfied and the number of positive cells were decreased but distributed in wide area. More cells were found near CA3 field in one week after injection, suggesting newly formed cells were migrated toward CA3 along hippocampal alveus.

Proliferation of neural stem cells in DG and CA1 7 days after ad libitum and dietary restriction

To investigate the effect of DR on neurogenesis in the adult brain, rats were maintained on either short term dietary restriction (DR) or *ad libitum* (AL) diets for 4 or 7 d, and BrdU-labelled cells were counted. Since fasting, average value of dropping weight was about 14.25 g for 4 d, and then, another 10.67 g for 3 d, which is about 5% and then 3% out of initial body weight.

BrdU labelled cells (green, observed from 4 d AL/DR) were commonly distributed in subgranular zone and granular cell layer in both AL and DR rat dentate gyrus as merged with propidium iodide (round shaped yellow signals). BrdU-labelled cell numbers were 7.02±0.67 in AL and 8.55±1.23 in DR respectively.

BrdU-labelled cells were also found in pyramidal cell layer and hippocampal alveus near CA1 subfield of hippocampus. After 4 d of DR, certainly more cells were immunostained in DR rats than in AL rats but statistical analysis showed no distinct difference in dentate gyrus and CA1 subfield. The average number of labelled cells were 4.29±0.43 in AL, 6.27±0.82 in DR (DG: AL (n=33)/DR (n=23), $*p<0.5$, CA1: AL(n=16)/DR(n=14), $**p<0.15$).

In dietary restricted rats for 7 d, BrdU-labelled cells were also observed to be widely distributed in subgranular zone and granular cell layer in AL and DR rats. The confocal images of DR group showed high rate of merged signal frequency compared to AL group (Fig. 2). In dentate gyrus, BrdU-labelled cell numbers in DR groups (12.31±0.97) were about two fold than in AL groups (6.65±0.75, AL (n=16)/DR (n=36), $*p<0.01$). BrdU-labelled cells were also found in pyramidal cell layer and hippocampal alveus near CA1 (Fig. 2c, d, g, h). The average number of BrdU-labelled cells in these area were more than two fold in DR (Fig. 3a, 4.27±0.67 in AL, 9.81±1.12 in DR, CA1: AL (n=15)/DR (n=31), $**p<0.01$).

To correct the systemic error of cell numbers counted in brain slices, we measured diameter of cell body and nucleus in dentate gyrus and pyramidal layer (Fig. 3b) and used the formula (P=(A){M/(L+M)} where P is the corrected number of cells per section, A is the uncorrected number of cells per section, M is the section thickness, and L is the average diameter of a cell in a section (Renfranz et al., 1991). Using the Z-stack in confocal microscope, we measured 3 axes (x-y-z) and statistically calculated the diameter of cell and nucleus (All group, n=30). The diameter of cell body was 15.17±0.80 μm and diameter of nucleus was 5.37±0.32μm in dentate gyrus. In the pyramidal layer of hippocampus, diameter of cell body was 18.22±1.22μm and diameter of nucleus was 6.92±0.60μm (Fig. 3b. *,

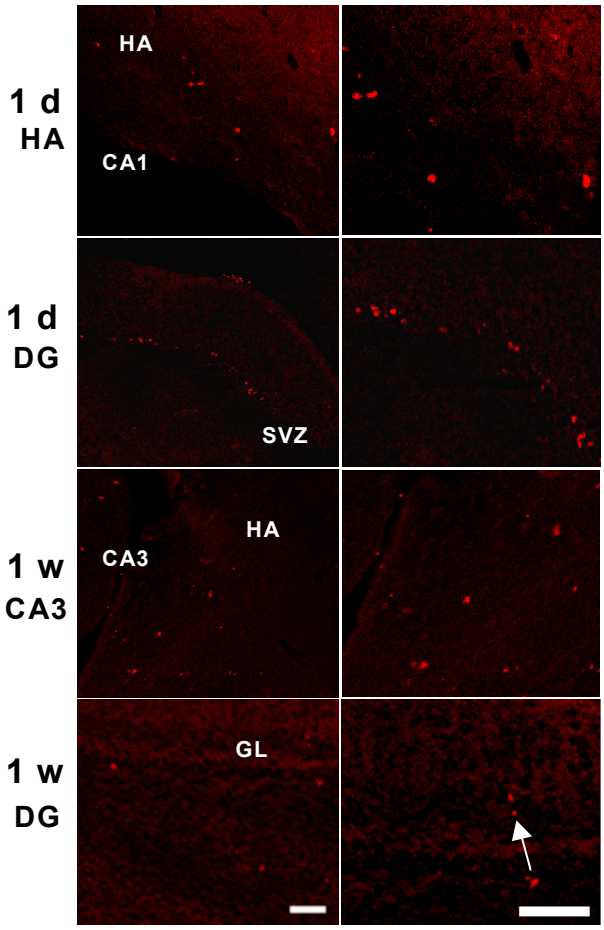

Fig. 1. The distribution of BrdU-labelled cells in dentate gyrus and hippocampal alveus in one d and one week after BrdU injection. BrdU-labelled cells were immunostained in hiipocampal alveus (HA) near CA1 subfield and subgranular zone (SGZ) one day after BrdU injection. One week after BrdU injection, cells were found to be scattered widely and migrated into CA3 subfield along hippocampal alveus in hippocampus and into granular cell layer (GL) in dentate gyrus (DG). All scale bars represent 20μm.

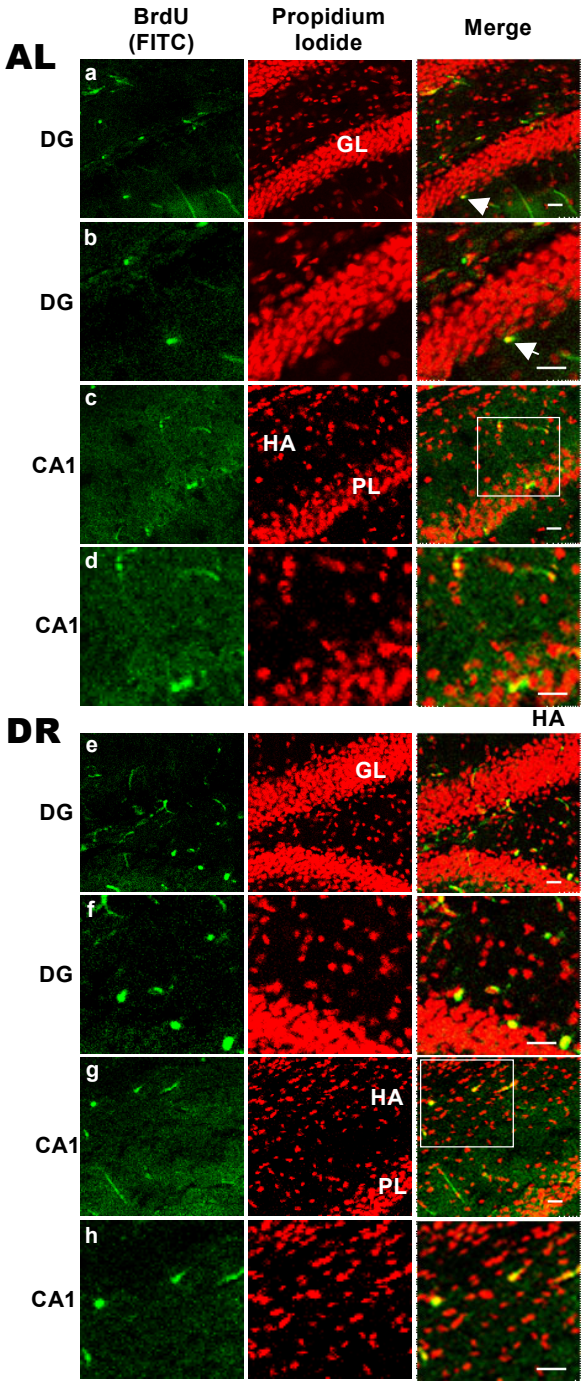

Fig. 2. Confocal images documenting newly generated cells (BrdU-labeled cells) in *ad libitum* (AL) and dietary restricted (DR) rat hippocampus. Newly generated cells (merged signal) are found in subgranular zone (SGZ), granular cell layer (GL) in dentate gyrus (DG) and hippocampal alveus (HA) and pyramidal cell layer (PL) in hippocampus of AL (a-d) and DR (e-h) rat brain. Sections were double-labeled with antibodies against BrdU (FITC, green) and propidium iodide (red). The migration of newly generated cells are shown in DG (arrowhead in a). All scale bars represent 20μm. Proliferation of neural stem cells were increased by DR.

$^{**}p < 0.05$). Cell diameter of granule cells in dentate gyrus seems shorter than that of pyramidal cells in CA1 field, probably because granular cells make tight dense layers.

These data indicates that proliferation of neural stem cells increased in dietary restricted rat hippocampus.

DISCUSSION

In the present study, we observed that neural stem cells were present in the adult rat brain and migrated as the time goes by in hippocampus as

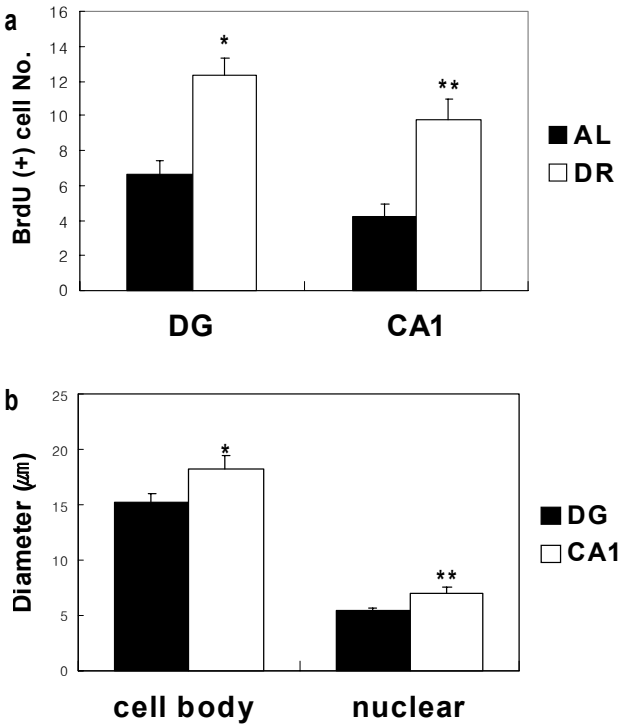

Fig. 3. The number of BrdU-labelled cells in *ad libitum* (AL) for 7 d and short-term dietary restriction (DR) in dentate gyrus (DG) and CA1 subfield in hippocampus. (a) The average number of BrdU-labelled cells in these area were more than two fold in DR (6.65±0.75 in AL (DG, n=16), 12.31± 0.97 in DR (DG, n=36), 4.27±0.67 in AL (CA1, n=15), 9.81 ±1.12 in DR (CA1, n=31), *,** $p < 0.01$). (b) The diameter of cell body and nucleus in DG and CA1. The diameter of cell body was 15.17±0.80μm and diameter of nucleus was 5.37±0.32μm in DG. In CA1, diameter of cell body was 18.22±1.22μm and diameter of nucleus was 6.92±0.60μm. The comparison of data that tested by ANOVA was different in DG and CA1 (*,** $p < 0.05$).

well as in dentate gyrus. We observed more BrdU-labelled cells in dentate gyrus but also present in hippocampal alveus, which means neural stem cells are dividing in both dentate gyrus and hippocampal alveus. There are many earlier studies reported that new neurons are generated in the dentate gyrus of the adult animals from birds to humans. We figured out that neural stem cells are generated in two areas in adult rat brain; dentate gyrus and hippocampal alveus.

One week after their proliferation, BrdU-labelled cell numbers decreased (in both 2-d injection group and 5-d injection group), suggesting some new cells die during their differentiation as they do during embryogenesis. Many BrdU-labelled cells were scattered widely. Some cells were found in the fissure and pyramidal cell layer, suggesting neural stem cells born in hippocampal alveus migrated following by hippocampal alveus into CA3 and into the fissure. Our previous study showed that neural stem cells transplanted to adult rat brain migrated following the hippocampal alveus which is the pathway as endogenous neuronal stem cells migrate during embryogenesis. Our results support these observations even though we can not figure out total movement of neural stem cells.

We observed that short-term dietary restriction (DR) increased the number of BrdU-labeled cells in adult rat hippocampus. In the previous studies, DR increased the survival of NPC progeny and reduces spontaneous death of newly generated cells in the hippocampus (Young et al., 1999; Lee et al., 2002). We found that short term DR increase proliferation of neural stem cells significantly in hippocampal alveus as well as in dentate gyrus. The major difference between our study and previous studies are period of DR. We made complete DR for 7 days contrary to other studies which made every-other-day DR for 3 mon. Complete DR may increase neural cell death, which may induce neural stem cell proliferation by producing neurotrophic factors. Further studies remain to elucidate how DR induces proliferation of neural stem cells.

In conclusion, we found that neural stem cells exist in the hippocampus of adult rat brain as well as in dentate gyrus, and they still divide and migrate follow the endogenous pathway. In addition, proliferation of neural stem cells were improved by the complete short-term DR.

ACKNOWLEDGEMENTS

This work was supported by fund from Korean Research Foundation (KRF 2000-015-DP0386).

REFERENCES

Adamchik Y, Frantseva MV, Weisspapir M, Carlen PL and Perez Velazquez JL (2000) Methods to induce primary and secondary traumatic damage in organotypic hippocampal slice cultures. *Brain Res Brain Res Protoc* 5:153-158.

Bruce-Keller AJ, Umberger G, McFall R and Mattson MP (1999) Food restriction reduces brain damage and improves behavioral outcome following excitotoxic and metabolic insults.

Ann Neurol 45:8-15.

Altman J and Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol* 124:319-335.

Duan W and Mattson MP (1999) Dietary restriction and 2-deoxyglucose administration improve behavioral outcome and reduce degeneration of dopaminergic neurons in models of Parkinson's disease. *J Neurosci Res* 15;57:195-206.

Duan W, Lee J, Guo Z and Mattson MP (2001) Dietary restriction stimulates BDNF production in the brain and thereby protects neurons against excitotoxic injury. J Mol Neurosci 16:1-12.

Dubey A, Forster MJ, Lal H and Sohal RS (1996) Effect of age and caloric intake on protein oxidation in different brain regions and on behavioral functions of mouse. *Arch Biochem Biophys* 333:189-197.

Lee J, Seroogy KB and Mattson MP (2002) Dietary restriction enhances neurotrophin expression and neurogenesis in the hippocampus of adult mice. *J Neurochem* 80:539-547.

Renfranz, P, Cunningham, MG and McKay RD (1991) Region-specific differentiation of hippocampal stem cell line HiB5 upon implantation into the developing mammalian brain. *Cell* 66:713-729.

Reynolds BA and Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 27;255:1707-1710.

Reynolds BA and Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* 175: 1-13.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD and Gage FH (2002) Functional neurogenesis in the adult hippocampus. *Nature* 28;415:1030-1034.

Young D, Lawlor PA, Leone P, Dragunow M and During MJ (1999) Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. *Nat Med* 5:448-453.

Yu ZF and Mattson MP (1999) Dietary restriction and 2-deoxyglucose administration reduce focal ischemic brain damage and improve behavioral outcome: evidence for a preconditioning mechanism. *J Neurosci Res* 15;57:830-839.

Zhu H, Guo Q and Mattson MP (1999) Dietary restriction protects hippocampal neurons against the death-promoting action of a presenilin-1 mutation. *Brain Res* 18;842:224-229.