RAPID REPORT

NEONATALLY BORN GRANULE CELLS NUMERICALLY DOMINATE ADULT MICE DENTATE GYRUS

R. MURAMATSU, Y. IKEGAYA, N. MATSUKI
AND R. KOYAMA*

Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Abstract—Hippocampal granule cells (GCs) are continuously generated in the subgranular zone of the dentate gyrus (DG) and functionally incorporated to dentate neural circuits even in adulthood. This raises a question about the fate of neonatally born GCs in adult DG. Do they exist until adulthood or are they largely superseded by adult-born GCs? To investigate this question, we examined the contributions of postnatally born GCs to the adult mouse DG. C57BL/6 mice were grouped in three different postnatal (P) ages (group 1: P0, group 2: P7, and group 3: P35) and received a daily bromodeoxyuridine (BrdU) injection for three consecutive days (P0/1/2, P7/8/9, and P35/36/37, respectively) to label dividing cells. At 6 months old, hippocampal sections were prepared from the animals and immunostained with anti-BrdU antibody and an antibody against the homeobox prospero-like protein Prox1, a marker of GCs. We defined BrdU- and Prox1-double positive cells as newborn GCs and analyzed their density and distribution in the granule cell layer (gcl), revealing that newborn GCs of each group still existed 6 months after BrdU injections and that the density of GCs born during P0-2 (group 1) was significantly higher compared with the other groups. Although the density of newborn GCs in the each group did not differ between male and female, the radial distribution of them in gcl showed some differences, that is, male newborn GCs localized toward the molecular layer compared with female ones in group 1, while to the hilus in group 2. These results suggest that GCs born in early postnatal days numerically dominate adult DG and that there exist sex differences in GC localizations which depend on the time when they were born. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: hippocampus, neurogenesis, postnatal, development, sex difference, Prox1.

Adult-born hippocampal granule cell (GC) normally migrate, survive (Rao et al., 2005) and are functionally incorporated into pre-existing neural circuits (van Praag et al., 2002), involved in certain types of learning and memory (Shors et al., 2001, 2002; Saxe et al., 2006). However, few studies focused on the fate of postnatally born GCs in the adult hippocampus. Rodent hippocampus begins to form in embryonic days (Altman and Bayer, 1990a,b) and most GCs are born 1 week after birth (Schlessinger et al., 1975) then migrate to the granule cell layer (gcl) (Altman and Bayer, 1990c) forming the dentate gyrus (DG), whereas the area of neurogenesis is restricted to the subgranular zone in adult DG with its scale getting smaller at least numerically in an age dependent manner (Kuhn et al., 1996). Here we have found that a good number of neonatally born GCs still exist in adult gcl with their density much higher than juvenile-born GCs and, interestingly, that their distribution in gcl showed sex differences.

The experimental design in the present study is outlined in Fig. 1. To label dividing cells in the early postnatal (P) DG, we have injected bromodeoxyuridine (BrdU; Sigma, St. Louis, MO, USA) s.c. to C57BL/6 mice (daily dose: 100 μg/g body weight) obtained from SLC (Shizuoka, Japan). Mice were grouped in three different P ages (group 1: P0, group 2: P7, and group 3: P35) and received a daily BrdU injection for three consecutive days (P0/1/2, P7/8/9, and P35/36/37, respectively) and were kept under temperature- and humidity-controlled conditions (23±1 °C, 50±10%, respectively). They were weaned at P35 then housed in cages separately by their sex with free access to food and water up to 6 months (P180) and then the animals were deeply anesthetized and perfused intracardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in ice-cold PBS. The coronal sections of mice brains represented by the image in Fig. 1B were prepared as previously described elsewhere (Kempermann et al., 2003) with minor modifications and hippocampal sections from both hemispheres/oronal section/one animal were analyzed in this study. Briefly, 30 μm sections were cut with a Leica CM 1800 freezing microtome (Bannockburn, IL, USA) and stored at −80 °C until the following immunohistochemical study. For detecting BrdU-labeled cells, the sections were pretreated with 2 N HCl for 30 min at 37 °C and washed in 0.1 M borate buffer (pH 8.5) for 10 min. They were incubated with 0.25% Triton X-100 and 2% goat serum in PBS at room temperature for an hour and then incubated at 4 °C overnight with mouse monoclonal anti-BrdU antibody (1:1000; Sigma) and rabbit polyclonal antibody against Prox1 (1:5000; Chemicon, Temecula, CA, USA), a specific marker of GCs (Pleasure et al., 2000; Encinas et al., 2006; Navarro-Quiroga et al., 2006). After extensive washes with PBS, samples were incubated with Alexa 488-labeled anti-rabbit IgG (1:1000; Invitrogen, Gaithersburg, MD, USA) and Alexa 594-labeled anti-mouse IgG (1:1000; Invitrogen). They were imaged...
with a Bio-Rad MRC-1024 confocal system (Richmond, CA, USA) with a 20× objective (Nikon, Tokyo, Japan) and reconstructed to three-dimensional images using ImageJ to confirm colabeling of BrdU and Prox1 (Fig. 1C). BrdU- and Prox1-colabeled cells were defined as GCs born at the time of BrdU injection, and their density and distribution were examined in gcl. To investigate whether the density and distribution of newborn GCs in entire hippocampus were represented by the single coronal section per animal as described above, we investigated the difference of the density and distribution between every tenth coronal series section (30 μm) throughout the entire hippocampus of a P10 mouse. There was no significant difference in the density (P>0.05, Smirnov-Grubbs’ test, n=5 series sections per hippocampus) and the lateral and radial distribution (P>0.05, Kolmogorov-Smirnov test, n=5 series sections per hippocampus) of newborn GCs throughout hippocampus from both hemispheres. These results indicate that the density and distribution of newborn GCs analyzed in the representative coronal sections used in the present study (Fig. 1B) reflect those of entire hippocampus. For the cell survival assay (Fig. 1E, F), mice in each group were killed 1 day after the last injection of BrdU and rostrocaudal hippocampal sections were prepared and immunostained as described above. The density of BrdU- and Prox1-colabeled cells was investigated in gcl and the hilus of P3 and P10 mice and only in gcl of P38 and 6-month-old mice in each group because there were a large number of migrating newborn GCs in the hilar region of P3 and P10 hippocampal sections but no such cells found in the sections prepared from P38 and 6-month-old mice (n=12–16 sections from six to eight mice). All experimental procedures conformed to the University of Tokyo guidelines concerning the care and use of animals for minimizing the number of animals used and their suffering.

**Fig. 1.** Experimental procedures. (A) C57BL/6 mice were subgrouped to different P ages and received daily s.c. injection of BrdU for three consecutive days to label dividing cells (group 1: P0/1/2; group 2: P7/8/9; group 3: P35/36/37). They were killed at 6 months old (P180). (B) The image of mouse brain coronal section, section 19 obtained from the Mouse Brain Library (http://www.mbl.org/) which corresponded to the section we used in this study. (C) The colabeling of BrdU (magenta) and the GC marker Prox1 (green) was confirmed by confocal three-dimensional reconstruction in the gcl and the cells double positive for both markers were defined as newborn GCs. Arrowheads indicate P0/1/2-born GCs in gcl of a 6-month-old mouse. Scale bar=10 μm. (D) The density of BrdU-labeled GCs in gcl of 6-month-old mice in each group (n=12 hippocampal sections from six mice in group 1 and group 2 respectively and n=16 sections from eight mice in group 3). The density of BrdU-labeled GCs decreased depending on the period they were born. **P<0.01 versus P0/1/2, ##P<0.01 versus P7/8/9; Tukey’s test after ANOVA. (E) Mice in each group were killed 1 day after the last injection of BrdU and rostrocaudal hippocampal sections were prepared and immunostained as described above. The density of BrdU- and Prox1-colabeled cells was investigated in gcl and the hilus of P3 and P10 mice and only in gcl of P38 and 6-month-old mice (n=12–16 sections from six to eight mice). All experimental procedures conformed to the University of Tokyo guidelines concerning the care and use of animals for minimizing the number of animals used and their suffering. **(F) The survival rate of postnatally born GCs at 6 months in each group was calculated as follows: (the postnatally born GC density at 6 months in group 1, group 2, and group 3, respectively/the newborn GC density at P3 (group 1), P10 (group 2) and P38 (group 3), respectively)×100 (%). The survival rate between each group showed no significant differences (n=12 hippocampal sections from six mice in each group, P>0.05; Tukey’s test after ANOVA). Data are mean±S.E.M.
Fig. 2. Neonatally born GCs dominate the adult gcl. (A) A confocal image of the right hemisphere DG from a group 1 mouse immunostained for BrdU (magenta) and Prox1 (green). P0/1/2-born GCs still remained in gcl 6 months after the last BrdU injection. Scale bar=100 μm. (B) A schematic illustration of gcl in Fig. 2A with BrdU-labeled GCs represented as solid circles. (C) A plot diagram of gcl made from Fig. 2B with a tip of the upper blade laterally to the right (lateral distribution rate: 1) and the ml radially to the top (radial distribution rate: 1) that demonstrates the distribution of BrdU-labeled GCs (solid circles). The histograms above and on the right of the square diagram indicate a frequency of GC appearance (% of total GCs) in the lateral and radial direction, respectively. Lateral (D) and radial (E) distribution of BrdU-labeled GCs with their density normalized by the average cell density of each group shown in Fig. 1D. Overwhelming number of P0/1/2-born GCs located in the middle and outer gcl compared with the other groups (E) but there were not such differences in the lateral distribution (D). Indeed, the cumulative frequency of BrdU-labeled GCs showed significant differences in the radial direction (G) between groups 1 and 2 (P<0.01) and between groups 2 and 3 (P<0.05), while no significant differences in the lateral direction (F) were observed between three groups (Kolmogorov-Smirnov test). Data are mean±S.E.M. of each of 12–14 hippocampal sections.
First of all, there existed many postnatally born GCs throughout gcl even at 6 months (Fig. 2A, B); the earlier BrdU was injected, the higher the density of them in gcl got (Fig. 1D). The overall density of P0/1/2-born GCs (2.66 × 10^4 ± 0.34 × 10^4 cells/mm^3; mean ± S.E.M.) was about twice as high as that of P7/8/9-born GCs (1.19 × 10^4 ± 0.19 × 10^4 cells/mm^3; mean ± S.E.M.) and more than nine times as high as that of P35/36/37-born GCs (0.28 × 10^4 ± 0.06 × 10^4 cells/mm^3; mean ± S.E.M.). For estimation of the total volume of gcl and the total number of GCs, coronal series sections (30 μm) were obtained throughout the entire hippocampus from 6-month-old mice (n = 3) and every tenth section was stained with 0.1% Cresyl Violet acetate. The total gcl volume was determined by summing the traced GC areas for each section multiplied by the distance between sections sampled. The total number of GCs was determined by multiplying GC number counted in fields within gcl in 30 × 30 × 30 μm sample volumes by the total volume of gcl. According to the gcl volume of 6-month-old C57BL/6 mice (0.31 ± 0.01 mm^3; mean ± S.E.M.), the total number of newborn GCs in gcl at 6 months was estimated in each group: P0/1/2, 0.83 × 10^6 cells; P7/8/9, 0.37 × 10^6 cells; P35/36/37, 0.09 × 10^6 cells. These values corresponded to 3.17%, 1.41%, and 0.35%, respectively, of the estimated total GC number of age-matched P0/1/2-born GCs in gcl. To determine the survival rate of postnatally born GCs at 6 months in each group, we killed animals 1 day after the final injection of BrdU (Fig. 1E) and investigated the density of newborn GCs. The density significantly decreased in an age-dependent manner: P0/1/2-born GCs at P3, 6.14 × 10^3 ± 2.05 × 10^3 cells/mm^3; P7/8/9, 1.20 × 10^3 ± 0.22 × 10^3 vs. 1.18 ± 0.05 × 10^4 (male vs. female, mean ± S.E.M. cells/mm^3). There were no significant sex differences in lateral distribution of newborn GCs in each group (Fig. 3A–C) whereas it was significantly shifted toward ml in male P0/1/2-born GCs and female P7/8/9-born GCs compared with the opposite sex (Fig. 3D, E). This kind of difference was not observed in the radial distribution of P35/36/37-born GCs. These results suggest that there exists the sex dependent regulation of survival, proliferation, and migration of newborn GCs that results in the difference of their distribution in adult gcl.

Our study showed that a number of postnatally born GCs survive even in the adult with their density highest in animals which had received BrdU injection during P0-2 (Fig. 1D). The density of neonatally (P0/1/2 and P7/8/9) born GCs in adult gcl was significantly higher even if compared with those born during the juvenile period (P35/36/37). Hippocampal neurogenesis in aged (21-month-old) rat gcl decreased in density to ~10% of that in 6-month-old one (Kuhn et al., 1996). In C57BL/6 mice, as much as two-thirds of adult-generated cells in DG died within 4 weeks (Kempermann et al., 1997) and assumed total number of GCs reached a plateau at 6 months (Kempermann et al., 1998). With our present study, it is indicated that postnatally born GCs, especially neonatally born ones, dominate adult mice gcl at least numerically. Further, according to some reports (Meschi et al., 2006; Saxe et al., 2006), not all hippocampal functions, including certain types of learning and memory, were associated with adult hippocampal neurogenesis. Now it is clear that more careful insight into not only adult neurogenesis but P neurogenesis is required to appreciate the function of adult hippocampus. One of the next questions to be addressed is the differences and relationships between postnatally born GCs and adult-born GCs in the adult hippocampus. The localization difference of GCs in gcl (earlier-born GCs to the outer gcl and later-born GCs to the inner gcl; Fig. 2E, G) might hold the key by outputting distinct signals which reflect the various layer specific neural inputs to GCs in dentate networks.

We further found the sex differences in the radial distribution rate of neonatally (P0/1/2 and P7/8/9) born GCs in gcl (Fig. 3D–F), but at this moment, we cannot conclude which steps in neurogenesis (like the migration rate, the local proliferation rate, or the local survival rate and so on) the sex difference affected, not to mention the factors that induced the sex differences. Considering the study reporting sex differences in the dendritic morphology of neonatal (P15-16) and prepubescent (P45-56) guinea-pig GCs (Bartesaghi et al., 2003), there seem to exist some sex-dependent factors which affect the development of GCs in the early life hippocampus. In the present study, rat pups were weaned at P35, and thus, possible effects of sex interactions on the difference of newborn GC localiza-
tion cannot be excluded because rodents are known to be sexually mature around 3 weeks after birth.

The fact that a large number of early postnatally born GCs still survive in adult DG involves important meanings from a pathological viewpoint because it implies that malformations of DG or GC itself induced in early P days by a number of factors such as hormones, growth factors, transmitters, stresses, environmental cues, and epileptic seizures etc., remain in adult DG and they may cause malfunction of the adult hippocampus. Especially, the authors are interested in the relationship of postnatally born GCs and the ectopic GCs which are observed in the patients of temporal lobe epilepsy (Houser, 1990). In a series of precise electrophysiological and anatomical experiments using rat models of temporal lobe epilepsy, Scharfman and colleagues (2007) have revealed that ectopic GCs, which incorrectly emerge in the hilus, have the synchronous epileptiform burst discharges with CA3 pyramidal cells (for review, see Scharfman et al., 2007). To date, ectopic GCs have been observed and studied in adult rat models of the disease when the hippocampal neurogenesis is restricted to the subgranular zone. Here we hypothesize that the early life seizure, including febrile seizure, disrupts the normal migration and differentiation of newborn GCs and detains them in the hilus resulting in an emergence of ectopic GCs in adulthood.

Finally we would like to mention based on the present study that more information about early P neurogenesis and development of hippocampus should be addressed because its function or malformation in adulthood might be destined by postnatally born GCs that remain for a long time, even if some newborn GCs are continuously added throughout adulthood.

Acknowledgments—The authors thank Ryuji X. Yamada for animal care and daily discussions. This work was supported in part by a Grant-in-Aid for Science Research (No. 18890052) and a Grant-in-Aid for JSPS Fellows from the Japan Society for the Promotion of Science.

REFERENCES


(Accepted 27 June 2007)
(Available online 17 July 2007)