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# Status Epilepticus During Old Age is not Associated With Enhanced Hippocampal Neurogenesis

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## Abstract

Increased production of new neurons in the adult dentate gyrus (DG) by neural stem/progenitor cells (NSCs) following acute seizures or status epilepticus (SE) is a well known phenomenon. However, it is unknown whether NSCs in the aged DG have similar ability to upregulate neurogenesis in response to SE. We examined DG neurogenesis after the induction of continuous stages III-V seizures (SE) for over 4 h in both young adult (5-months old) and aged (24-months old) F344 rats. The seizures were induced through 2–4 graded intraperitoneal injections of the excitotoxin kainic acid (KA). Newly born cells in the DG were labeled via daily intraperitoneal injections of the 5′-bromodeoxyuridine (BrdU) for 12 days, which commenced shortly after the induction of SE in KA-treated rats. New cells and neurons in the subgranular zone (SGZ) and the granule cell layer (GCL) were analyzed at 24 h after the last BrdU injection using BrdU and doublecortin (DCX) immunostaining, BrdU-DCX and BrdU-NeuN dual immunofluorescence and confocal microscopy, and stereological cell counting. Status epilepticus enhanced the numbers of newly born cells ($BrdU^+$ cells) and neurons ($DCX^+$ neurons) in young adult rats. In contrast, similar seizures in aged rats, though greatly increased the number of newly born cells in the SGZ/GCL, failed to increase neurogenesis due to a greatly declined neuronal fate-choice decision of newly born cells. Only 9% of newly born cells in the SGZ/GCL differentiated into neurons in aged rats that underwent SE, in comparison to the 76% neuronal differentiation observed in age-matched control rats. Moreover, the number of newly born cells that migrate abnormally into the dentate hilus (i.e., ectopic granule cells) after SE in the aged hippocampus is 92% less than that observed in the young adult hippocampus after similar SE. Thus, SE fails to increase the addition of new granule cells to the GCL in the aged DG, despite a considerable upregulation in the production of new cells, and SE during old age leads to much fewer ectopic granule cells. These results have clinical relevance because earlier studies have implied that both increased and abnormal neurogenesis occurring after SE in young animals contributes to chronic epilepsy development.

### Keywords

adult neurogenesis; aging; 5′-bromodeoxyuridine; DG; dentate neurogenesis; doublecortin; kainic acid; neural stem cells; rat; stem cell proliferation; stem cell differentiation



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## INTRODUCTION

Neurogenesis in the DG of the hippocampus takes place throughout life in nearly all mammals (Altman and Das, 1965; Kuhn et al., 1996; Gould et al., 1997; Eriksson et al., 1998; Gould et al., 1999 a; Kornack and Rakic, 1999). Numerous studies validate that newly added neurons (i.e., dentate granule cells) get integrated into the hippocampal circuitry and participate in hippocampal-dependent learning and memory functions and mood (Gould et al., 1999b; van Praag et al., 1999, 2002, 2005; Feng et al., 2001; Shors et al., 2001, 2002; Drapeau et al., 2003, 2007; Santarelli et al., 2003; Bruel-Jungerman et al., 2005; Aimone et al., 2006; Leuner et al., 2006; Zhao et al., 2006; Ge et al., 2007; Kee et al., 2007; Toni et al., 2007). It is also well known that the amount of neurogenesis in the adult DG is not stagnant, as it fluctuates noticeably depending upon alterations in the DG milieu. Studies reveal that increased levels of neurotrophic factors (such as fibroblast growth factor-2 [FGF-2], brain-derived neurotrophic factor [BDNF], insulin-like growth factor-1 [IGF-1] and vascular endothelial growth factor [VEGF]), enhanced physical activity, caloric restriction, environmental enrichment, and increased neural activity are positive regulators of neurogenesis (Kempermann et al., 1998, 2002; Gould, 1999; Nilsson et al., 1999; van Praag et al., 1999, 2005; Lichtenwalner et al., 2001; Jin et al., 2002, 2003; Sun et al., 2003, 2006; Mirescu et al., 2004; Sairanen et al., 2005; Rai et al., 2007). On the other hand, aging is associated with dramatically decreased neurogenesis (Kuhn et al., 1996; Nacher et al., 2003; Rao et al., 2005, 2006a; Hattiangady et al., 2007; Hattiangady and Shetty, 2008). Furthermore, DG neurogenesis in the young brain exhibits notable plasticity to injury through considerable upregulation in the production of new neurons (Liu et al., 1998; Jin et al., 2001, 2004). While the latent benefits or adverse effects of increased neurogenesis after seizures or brain injury are still being examined (Parent, 2003; Jessberger et al., 2007a; Pekcec et al., 2007; Raedt et al., 2007; Scharfman and Gray, 2007; Shetty and Hattiangady 2007), studies in some brain injury models suggest that this plasticity may be valuable for diminishing impairments in cognitive functions after brain injury (Kleindienst et al., 2005; Sun et al., 2007).

Acute seizures or status epilepticus (SE) produced through administration of chemoconvulsants enhance neurogenesis to a great extent in the young adult DG (Bengzon et al., 1997; Parent et al., 1997; Gray and Sundstrom, 1998; Hattiangady et al., 2004; Parent et al., 2006; Gong et al., 2007). It has been demonstrated that SE in young adult animals induces an initial, transitory proliferative surge in the SGZ with the number of new neurons increasing severalfold during the first few weeks after SE. This is likely due to both direct effects of seizures and upregulation of several neurotrophic factors in the young adult hippocampus after seizure-induced injury. However, it is unknown whether aging alters the response of neural stem/progenitor cells (NSCs) in the DG to SE. This is an important issue because the incidence of SE is much higher during old age (Towne, 2007). Moreover, characterization of the postlesion plasticity indicate that aging diminishes the synaptic reorganization and upregulation of neurotrophic factors following injury in the hippocampus (Schauwecker et al., 1995; Woods et al., 1998; Shetty and Turner, 1999; Shetty et al., 2004). Furthermore, studies suggest that increased neurogenesis and enhanced ectopic migration of newly born granule cells into the dentate hilus after acute seizures or SE likely contributes to or exacerbates the development of chronic epilepsy after SE (Scharfman et al., 2000, 2002, 2003; Jung et al., 2004; Pierce et al., 2005; McCloskey et al., 2006; Parent, 2007; Scharfman and Gray, 2007). From these perspectives, and because the aged population is generally more vulnerable to acute seizures and SE resulting from stroke and brain tumors, and the likelihood for chronic epilepsy in humans rises with aging (Eisenschenk and Gilmore, 1999; LaRoche and Helmers, 2003), it is imperative to investigate age-related changes in the reaction of DG neurogenesis to SE.

We rigorously examined whether the capability of the DG to augment neurogenesis in response to acute seizures is preserved during aging. We investigated DG neurogenesis after the induction of continuous stages III–V seizures (SE) for over 4 h in both young adult (4-months old) and aged (24-months old) F344 rats. The seizures were induced through 2–4 graded intraperitoneal injections of the excitotoxin kainic acid (KA). Newly born cells in the DG were labeled via daily intraperitoneal injections of the 5′-bromodeoxyuridine (BrdU) for 12 days, which commenced shortly after the induction of SE in KA-treated rats. New cells and neurons in the subgranular zone (SGZ) and the granule cell layer (GCL) were analyzed at 24 h after the last BrdU injection using BrdU and doublecortin (DCX) immunostaining, BrdU-DCX and BrdU-NeuN dual immunofluorescence and confocal microscopy, and the optical fractionator cell counting method.

## EXPERIMENTAL PROCEDURES

### Animals

Animals used in this study were obtained from the National Institutes of Aging colony of F344 rats maintained at Harlan Sprague-Dawley (Indianapolis, IN). Four groups of rats were used for this study. These include intact young adult rats (4-months old, $n = 6$), intact aged rats (24-months old, $n = 6$), young adult rats receiving graded intraperitoneal injections of KA ($n = 8$), and aged rats receiving graded intraperitoneal injections of KA ($n = 8$). The animals were individually housed in an environmentally controlled room (~23°C) with a 12:12-h light-dark cycle, and were given food and water ad libitum. All experiments were performed as per the animal protocol approved by the institutional animal care and use committee of the Duke University Medical Center and the animal studies subcommittee of the Durham Veterans Affairs Medical Center.

### Induction of SE in Young Adult and Aged Rats

Rats were injected intraperitoneally with KA (3.0 mg/kg body weight) every hour for 2–4 h for induction of acute seizures and SE (Rao et al., 2006b). This protocol for F344 rats is adapted from the procedure developed by Hellier et al. (1998) for Sprague-Dawley rats. A vast majority of KA-treated young adult rats began to have apparent motor seizures by the third or fourth injection whereas aged rats exhibited full blown motor seizures by the second injection. Thus, each young adult animal received a total KA dose of 9–12.0 mg/kg b.w. whereas each aged animal received a total dose of 6 mg/kg b.w. in this study. Seizures were scored as per the modified Racine's scale (Racine, 1972; Ben-Ari, 1985; Hellier et al., 1998). The motor seizures were characterized by unilateral forelimb clonus with lordotic posture (stage III seizures), bilateral forelimb clonus and rearing (stage IV seizures), and bilateral forelimb clonus with rearing and falling (stage V seizures). The onset of status was defined as the first stage V seizure that did not decline after several minutes. Most young adult and aged animals receiving the above doses of KA exhibited continuous stages III–V motor seizures for 4 h after the onset of the status. We quantified the number of motor seizures (stages III–V) and the duration of individual seizures for each hour during the period of SE. During and after SE, rats were housed in a cage with soft foam covered lid to prevent them from bouncing against the metallic roof of the cage. These rats were given moistened rat chow and a 10 ml injection of lactated Ringer's every day for 3–5 days after SE. To avoid confounds, rats exhibiting seizures for less than 4 h (1–2 out of 8 rats in both age groups) were excluded from further analyses in this study. Although we did not administer any antiepileptic drug (such as diazepam) to stop seizures at 4 h after the SE, rats in both groups exhibited only occasional stages III–V seizures during the 5th hour after SE and were free of stages III–V seizures during the 6th hour after SE.

### Administration of BrdU for Labeling Newly Born Cells in the DG and Harvesting of Brain Tissues

Both intact rats and rats that underwent KA-induced SE received daily intraperitoneal injections of BrdU (Sigma, St Louis, MO) for 12 consecutive days at a dose of 100 mg/kg body weight. In rats that underwent KA-induced SE, daily BrdU injections were commenced on the day of SE and ended on post-SE day 12. In all groups, rats were fatally anesthetized with halothane and perfused with 4% paraformaldehyde at 24 h after the last of twelve daily BrdU injections and brains collected for histological analyses of newly born cells and neurons that are added to the different regions of the DG over a period of 12 days.

### Analyses of Hippocampal Cytoarchitecture After KA Induced SE

The brains were removed, postfixed in 4% paraformaldehyde for 16 h at 4°C and cryoprotected in 30% sucrose solution in phosphate buffer (PB). Thirty-micrometer thick cryostat sections were cut coronally through the entire antero-posterior axis of the hippocampus and collected serially in PB. Every 15th section through the hippocampus was selected in each of the animals and processed for Nissl staining. Nissl staining demonstrated hippocampal cytoarchitecture in both control and KA-treated animals. In rats receiving KA, this analysis determined the extent of hippocampal injury following KA-induced SE in young adult and aged rats.

### BrdU and DCX Immunohistochemistry

Serial sections (every 15th) through the entire hippocampus were selected in each animal belonging to different groups and processed for BrdU immunostaining using a monoclonal antibody to BrdU (Roche diagnostics; Indianapolis, IN). Another series (every 15th) of sections were processed for DCX immunostaining using a polyclonal antibody to DCX (Santa Cruz Biotechnology; Santa Cruz, CA) using avidin-biotin complex method described in our earlier reports (Rao and Shetty, 2004; Rao et al., 2005). The visualization of the peroxidase reaction was done using diaminobenzidine as the chromogen for BrdU and Vector gray (Vector) as the chromogen for DCX. The immunostained sections were mounted on gelatin-coated slides, air-dried, counter-stained with hematoxylin, dehydrated, cleared, and cover slipped.

### Quantification of the Total Number of $BrdU^+$ Cells and $DCX^+$ Neurons in the DG

The $BrdU^+$ cells in the dentate SGZ (two-cell thick region from the inner margin of the dentate GCL) and the GCL were counted in every 15th section through the entire antero-posterior extent of the hippocampus, in every animal belonging to each of the four groups ($n$ = 5/Group) killed at 24 h after the last of twelve daily BrdU injections. As an additional measure of neurogenesis, $DCX^+$ neurons in the SGZ-GCL and the dentate hilus (DH) were counted separately in every animal belonging to each of the four groups ($n$ = 5/Group). Although we included 8 animals per age-group for inducing SE, because of the deteriorating health (such as weight loss and depression) of some rats during the immediate post-SE period, 1–2 animals in each group had to be euthanized before the desired endpoint (i.e., 13 days post-SE). Additionally, 1–2 animals in each group had to be excluded as they did not develop continuous SE for 4 h. Nevertheless, five animals that underwent continuous SE for 4 h were available in both age groups at the desired end-point for quantitative analysis, which prompted quantification of data from 5 animals in every group. The StereoInvestigator system (Microbrightfield Inc., Williston, VT), consisting of a color digital video camera (Optronics Inc., Muskogee, OK) interfaced with a Nikon E600 microscope, was used for counting of cells, as described in our previous reports (Rao and Shetty, 2004; Rao et al., 2005, 2006a).

### Measurement of Neuronal Differentiation of Newly Born Cells via DCX and BrdU Dual Immunostaining

To measure the percentages of newly born cells ($BrdU^+$ cells) that differentiate into neurons in the SGZ-GCL, representative sections ($n = 4$) from animals belonging to all groups were processed for DCX and BrdU dual immunofluorescence. The sections were first processed for DCX immunofluorescence using primary antibody against DCX (goat polyclonal, Santa Cruz), biotinylated horse antigoat IgG (secondary antibody from Vector) and streptavidin fluorescein (Molecular Probes), which gave green fluorescence to DCX in soma and dendrites (Hattiangady and Shetty, 2008). Then, the sections were washed thoroughly and processed for BrdU immunofluorescence using methods described elsewhere (Hattiangady et al., 2007). Cells that exhibited BrdU and DCX coexpression were identified using a Nikon E600 Fluorescence microscope. The fractions of $BrdU^+$ cells that express DCX were then quantified by examination of individual $BrdU^+$ cells at 400× ($n = 4$/age group) in a confocal laser scanning microscope (LSM 410). For this, 1-lm thick optical Z-sections were sampled from different regions of the SGZ-GCL in all groups and the images were analyzed using LSM image browser.

### Analyses of Mature $NeuN^+$ Neurons Among Newly Born Cells

To visualize the fractions of $BrdU^+$ cells that express the mature neuronal marker NeuN, representative sections ($n = 4$) from all groups were processed for BrdU and NeuN dual immunofluorescence staining. The sections were first processed for various BrdU preincubation treatments (Rao and Shetty, 2004; Rao et al., 2005, 2006a), washed in Tris-buffered saline (TBS), blocked in normal serum, incubated in a cocktail solution containing rat anti-BrdU (1:50, Accurate Chemicals) and mouse anti-NeuN (1:1,000) and washed in TBS. The sections were then treated with a mixture of goat anti-mouse IgG tagged with Alexa Fluor 488 and biotinylated rabbit antirat IgG (Vector), washed in TBS, incubated in streptavidin Texas red, rinsed in TBS, and cover slipped with slow fade/antifade mounting medium (Molecular Probes). Cells that exhibited BrdU and NeuN coexpression were identified using a Nikon E600 Fluorescence microscope. Fractions of $BrdU^+$ cells that express NeuN were then quantified by examination of individual $BrdU^+$ cells at 400× in confocal laser scanning microscope (LSM 410). For this, 1-μm thick optical Z-sections were sampled from different regions of the SGZ-GCL in all groups and the images were analyzed using LSM image browser.

### Statistical Analyses

For every parameter, the average value was first calculated separately for each animal before the means and standard errors were determined for the total number of animals included per group. The values (Mean ± S.E.M.) from different groups of animals were compared using one-way analysis of variance (ANOVA).

## RESULTS

### Acute Seizures and Hippocampal Neurodegeneration After Graded Intraperitoneal Injections of KA

The seizures were induced in young adult (4-months old) and aged (24-months old) F344 rats through 2–4 graded intra-peritoneal injections of the excitotoxin kainic acid (KA; 3 mg/kg bw/h). Rats were observed for the presence of seizures after every KA injection. In the young adult group, continuous seizures were observed after 3–4 injections of KA. In contrast, in aged animals, continuous seizures were observed after just two injections of KA, suggesting that the vulnerability of aged rats to seizures is greater. This is exemplified by the induction of SE with lower cumulative dose (6 mg/kg b.w.) of KA in aged rats, in

comparison to young adult rats which needed 9–12 mg/kg. b.w. of KA for induction of SE. Increased susceptibility of aged rats to seizures observed here is consistent with several recent studies on aging and seizures (Darbin et al., 2004; Liang et al., 2007). Both young adult and aged rats exhibited stages III–V motor seizures continuously for 4 h. Seizures were characterized by unilateral forelimb clonus (stage III), bilateral forelimb clonus (stage IV), and bilateral forelimb clonus with rearing and falling (stage V). Although we did not administer any antiepileptic drug (such as diazepam) to stop seizures at 4 h after the SE, rats in both groups exhibited only occasional stages III–V seizures during the 5th hour after SE and were free of stages III–V seizures during the 6th hour after SE. Measurement of the frequency and duration of seizures during the 1st, 2nd, 3rd, and 4th hour of SE revealed comparable frequency and duration of seizures between young adult and aged animals (Fig. 1). The number of stages III–V seizures per hour during SE in both groups varied from ~7 during the first hour, ~12 during the second hour, ~18 during the third hour, and ~20 during the 4th hour (Fig. 1A). The duration of individual seizures at different hours of SE in both groups varied from 25 to 26 s during the first hour, 43–44 s during the second hour, 49–50 s during the third hour, and 51–55 s during the 4th hour (Fig. 1B).

In both age groups, KA-induced SE caused considerable neurodegeneration in the hippocampus (Fig. 2) and extrahippocampal regions such as the thalamus, the entorhinal cortex (EC), the piriform cortex and amygdala (data not illustrated), when examined at 13-days post-SE. In the hippocampus of both young adult and aged animals, significant bilateral neuronal cell loss was observed in the dentate hilus (Figs. 2B2, D2), CA1 pyramidal cell layer (Figs. 2B3, D3), and CA3c sub region of the CA3 pyramidal cell layer (Figs. 2B4, D4). Some cell loss (indicated by thinning of the cell layer) was also observed in the CA3a and CA3b sub regions (Figs. 2B1, D1) in comparison to the age-matched hippocampus of naïve control animals (Figs. 2A1, C1). However, the dentate granule cell layer did not exhibit any apparent cell loss in both age groups (Figs. 2B1, D1). Thus, KA-induced SE causes similar pattern of injury in the hippocampus of young adult and aged rats.

## Age-Related Changes in the Extent of Addition of New Cells to SGZ-GCL After SE

Age-related changes in the extent of addition of new cells to the SGZ-GCL following SE were assessed through BrdU immunostaining of hippocampal sections from animals belonging to both young adult and aged groups killed at 24 h after the last of twelve daily BrdU injections (Fig. 3). In KA treated animals, the BrdU injections were administered during the post-SE days 1–12. In all groups, BrdU immunostaining revealed newly generated cells in the GCL and the SGZ throughout their antero-posterior extent. The distribution and numbers of newly born cells in the SGZ-GCL of intact young adult and aged rats have been described in our earlier report (Rao et al., 2005). The hippocampus of young adult rats that underwent SE exhibited a dramatic increase in the number of newly born cells (i.e., $BrdU^+$ cells) in the SGZ-GCL (Figs. 3B1, B2), in comparison to age-matched intact hippocampi (Figs. 3A1, A2). The degenerated regions (dentate hilus and the CA3 pyramidal cell layer) also showed a large number of $BrdU^+$ cells (Figs. 3B1, D1). The hippocampus of aged rats that underwent SE also showed increased density of $BrdU^+$ cells in SGZ-GCL (Figs. 3D1, D2) in comparison to age-matched intact hippocampus (Figs. 3C1, C2). This suggests that considerable upregulation in the numbers of newly born cells occurs in both young adult and aged animals after SE. Quantification of the number of new cells (i.e., $BrdU^+$ cells) added to the SGZ-GCL over a period of 12 days (i.e., during the post-SE days 1–12) revealed a marked increase in the addition of new cells in both young adult and aged hippocampi after SE ($P < 0.001$; Fig. 3E1). The overall increase is 2.6-folds in young adult rats and 5.9-folds in aged rats. Thus, in both young adult and aged animals, KA-induced SE leads to a dramatic increase in the addition of new cells to the dentate SGZ and GCL (Fig. 3E1).

### Age-Related Alterations in the Neuronal Fate-Choice Decision of Newly Born Cells After SE

Dual immunostaining for BrdU and DCX in tissues harvested at 24 h after the last of twelve daily BrdU injections revealed neurons among newly born cells ($BrdU^+$ cells) in the SGZ-GCL. The BrdU expression was found in the nucleus whereas DCX staining was observed in the soma and dendrites of newly born neurons (Fig. 4A1–B3). Doublecortin, a microtubule-associated phosphoprotein, is an excellent marker of immature neurons in the DG. Studies imply that DCX expression occurs within 3 h after birth in neuronally committed newly born cells (Kempermann et al., 2003) and persists for at least 2 weeks (Rao and Shetty, 2004). In naive animals, our previous analyses have demonstrated that aging does not alter the neuronal fate-choice decision of newly born cells in the SGZ-GCL, as comparable percentages of new cells differentiated into $DCX^+$ neurons in naive young adult as well as aged animals (Rao et al., 2005, 2006a). Interestingly, SE-induced injury did not affect the fate-choice decision of newly born cells in young adult animals, as fractions of newly born cells that differentiated into neurons were similar between naive animals (Mean ± standard error of mean (SEM) = [(75.8 ± 6.9)%] and animals that underwent SE [(68.0 ± 1.2)%] in this age group (Fig. 4C1). In contrast, in aged animals, the neuronal differentiation of newly born cells was (9.0 ± 0.4)% after SE, in comparison to neuronal differentiation of (76 ± 1.9)% newly born cells observed in intact animals ($P < 0.001$; Fig. 4C1). Thus, SE-induced hippocampal injury considerably reduces the neuronal fate-choice decision of newly born cells in aged animals, as much greater fractions of newly born cells differentiate into neurons in intact aged rats than aged rats that underwent SE.

### Age-Related Changes in the Expression of NeuN in Newly Born Cells After SE

Dual immunostaining for BrdU and the mature neuronal marker NeuN in hippocampal tissues collected at 24 h after the last of twelve daily BrdU injections revealed mature neurons among newly born cells ($BrdU^+$ cells) in the SGZ-GCL. The BrdU expression was found in the nucleus whereas NeuN staining was found in both nucleus and soma of newly born neurons (Figs. 5A1–B3). Expression of NeuN by newly born cells remained stable after SE in young adult animals, as fractions of newly born cells that differentiated into $NeuN^+$ neurons were similar between naive animals [(37.8 ± 0.7)%] and animals that underwent SE [(38.8 ± 1.4)%] in this age group (Fig. 5C1). In aged animals, the expression of NeuN by newly born cells decreased after SE, as the fraction of newly born cells that differentiated into $NeuN^+$ neurons was (6.4 ± 1.2)% after SE, in comparison to (14.3 ± 0.6)% observed in intact animals ($P < 0.001$; Fig. 5C1). Thus, SE-induced hippocampal injury considerably reduces the differentiation of newly born cells into $NeuN^+$ mature neurons in aged animals, as greater fractions of newly born cells differentiate into $NeuN^+$ neurons in intact aged rats than aged rats that underwent SE. Reduced fraction of $NeuN^+$ neurons among newly born cells in the intact aged hippocampus (in comparison to the intact young adult hippocampus) on the other hand is however due to delayed acquisition of NeuN by newly born cells in the aged hippocampus. This conclusion is based on several time-points of analyses performed after BrdU injections in our previous study using intact aged animals (Rao et al., 2005).

### SE-Induced Changes in the Production of $DCX^+$ New Neurons in Young Adult and Aged DG

We examined $DCX^+$ newly born neurons in the SGZ-GCL and the dentate hilus of both age groups of rats as an additional measure of the status of DG neurogenesis after SE (Fig. 6). An increased density of $DCX^+$ newly born neurons was observed after SE in both SGZ-GCL and the dentate hilus of young adult animals (Figs. 6B1, B2), in comparison to new neurons in respective regions of the age-matched intact animals (Figs. 6A1, A2). However, the density of $DCX^+$ newly born neurons in the SGZ-GCL of aged animals that underwent SE (Figs. 6D1, D2) was comparable to the new neuron density observed in respective regions of

age-matched intact animals (Figs. 6C1, C2). Although the dentate hilus of aged animals also showed a few $DCX^+$ neurons following SE (Figs. 6D1, D2, D4), the ectopic migration of newly born cells into the dentate hilus following SE was very conspicuous in young adult animals (Figs. 6B1, B2, B3), which is consistent with the earlier reports in SE models using young adult animals (Parent et al., 1997; Hattiangady et al., 2004). In the aged group, the overall density and pattern of distribution of $DCX^+$ neurons in the DG appeared dramatically less than that of $BrdU^+$ cells after SE, likely because only a very small fraction of newly born cells differentiated into neurons. Furthermore, a vast majority of $DCX^+$ neurons located in the SGZ-GCL of intact young adult rats exhibited the phenotype of differentiated granule cells with vertically oriented dendrites extending into the outer two-thirds of the dentate molecular layer (Figs. 6A2). In intact aged rats (Fig. 6C2), young adult rats that underwent SE (Fig. 6B2), and aged rats that underwent SE (Fig. 6D2, the $DCX^+$ neurons with vertically oriented dendrites reaching the outer two-thirds of the molecular layer were rarely seen. Furthermore, the morphology of $DCX^+$ neurons that have migrated ectopically into the dentate hilus following hippocampal injury was similar between the two age groups (Figs. 6B3, D4). These neurons exhibited horizontally oriented dendrites or dendrites that projected mostly towards the dentate hilus (i.e., basal dendrites). In addition, some of the newly born neurons that are located in the SGZ-GCL also exhibited long basal dendrites (Figs. 6B4, D3), as observed typically in SE models of epilepsy (Shapiro and Ribak, 2006; Ribak and Shapiro, 2006; Jessberger et al., 2007b).

By measuring the numbers of $DCX^+$ neurons in the SGZ-GCL and the dentate hilus of different groups the following results emerged. Consistent with the BrdU results described above and earlier studies, SE considerably increased the number of new neurons in the DG of young adult rats. In comparison to numbers in age matched intact rats, the overall increase was 2.2-folds in the SGZ-GCL (Fig. 6E1), 28-folds in the dentate hilus (Fig. 6E2) and 3-folds in the entire DG. However, SE did not increase the numbers of new neurons in the SGZ-GCL of aged rats (Fig. 6E1). The SGZ-GCL of intact aged rats exhibited 3,763 ± 190 new neurons and this number did not change significantly after SE (4,663 ± 473; $P > 0.05$). Although SE induced 2-fold increase in the numbers of new neurons in the dentate hilus of aged rats, the overall extent of increase seen in these rats was much less in comparison to 28-fold increase observed in young adult rats after similar SE (Fig. 6E2). When taken as a whole entity (i.e., by adding new neurons in the SGZ-GCL and the dentate hilus), the DG of aged rats did not exhibit increased number of new neurons after SE-induced hippocampal injury, unlike the DG of young adult rats.

Measurement of fractions of newly born neurons that are located in the dentate hilus revealed that, under intact conditions, only 3.5% of newly born neurons migrate into the dentate hilus in young adult rats. In contrast, in aged rats, nearly 20% of newly born neurons are located in the dentate hilus. Ectopic migration of newly born neurons into the dentate hilus increases to 34% of all newly born neurons (i.e., ~10-fold increase) following SE in young adult rats. In aged rats, ectopic migration of newly born neurons into the dentate hilus increases to 35% of all newly born neurons (i.e., ~1.7-fold increase) following SE. Thus, percentages of newly born cells that exhibit ectopic migration into the dentate hilus after SE are comparable between young adult and aged groups. However, because of no increases in the overall neurogenesis after SE, aged rats that underwent SE exhibit dramatically reduced number of ectopically placed newly born cells (1,620 ± 91) than young adult rats that underwent SE (21,015 ± 1,356).

## DISCUSSION

This quantitative study demonstrates for the first time that SE during old age does not increase the production of new neurons in the hippocampus. These results are intriguing

because earlier studies have consistently documented increased hippocampal neurogenesis after acute seizures or SE during young and adult age. Moreover, unchanged neurogenesis in response to SE during old age is a result of dramatically diminished neuronal fate-choice decision of newly born cells after SE, as production of new cells in the neurogenic niche (SGZ) of the hippocampus exhibited 5.9-fold increases after SE. Furthermore, unlike during young and adult age, SE during old age is associated with much fewer newly born, displaced granule cells in the dentate hilus. While fractions of newly born neurons that migrate ectopically into the dentate hilus after SE is comparable between young adult and aged groups (~35% of all new neurons), the actual number of newly born neurons that migrate aberrantly into the dentate hilus in the aged hippocampus is 92% less than that observed in the young adult hippocampus. This is due to production of much reduced number of neurons in the aged hippocampus than the young adult hippocampus after SE.

### Extent of Age-Related Changes in Seizure-Induced Neurogenesis

Increased hippocampal neurogenesis after acute seizures or SE is a well known phenomenon observed in multiple animal models of epilepsy using young adult or adult animals (Parent et al., 1997, 1998, 2006; Scott et al., 1998; Nakagawa et al., 2000; Overstreet-Wadiche et al., 2006; Jessberger et al., 2007a,b; Parent, 2007; Scharfman and Gray, 2007; Walter et al., 2007). Hippocampal neurogenesis also increases after an intracerebroventricular KA administration in anesthetized young adult rats (Gray and Sundstrom, 1998; Hattiangady et al., 2004, 2008). Consistent with these studies, KA-induced SE was associated with increased neurogenesis in the DG of young adult rats in this study. Seizure-induced increase in neurogenesis in this age group was associated with considerably enhanced addition of new cells to the SGZ-GCL, and neuronal fate-choice decision of newly born cells (68% of all new cells) equivalent to that found in the age-matched naive control animals (76% of all new cells). The overall rise in the addition of new cells over a period of 12 days after SE to SGZ-GCL was 2.6-folds based on BrdU labeling. On the basis of BrdU-DCX analyses at 24 h after the last of 12 daily BrdU injections, the addition of new neurons to the DG was increased by 2.3-folds between post-SE days 1 and 12 in the young adult hippocampus. Additional characterization of the status of the neurogenesis through measurement of the total number of $DCX^+$ new neurons at 13-days post-SE also demonstrated 2.2-folds increase in the addition of new neurons. On the contrary, KA-induced SE was not associated with increased neurogenesis in the SGZ-GCL of aged rats. Interestingly, unchanged neurogenesis after seizures in this age group was associated with 5.9-folds increase in the production of new cells in the SGZ-GCL. However, even this dramatic increase in the production of new cells did not translate into increased addition of new neurons due to considerably reduced neuronal fate-choice decision of newly born cells (only 9% of all new cells) in contrast to much greater neuronal differentiation found in the age-matched naive control animals (76% of all new cells). On the basis of both BrdU-DCX analyses at 24 h after the last of 12 daily BrdU injections and analyses of the status of the neurogenesis through counting of $DCX^+$ new neurons at 13 days post-SE, there was clearly no increase in the addition of new neurons to the aged SGZ-GCL after SE. Measurement of $DCX^+$ neurons in the dentate hilus demonstrated that the ectopic migration of newly born neurons after SE was increased by 10-folds in the young adult hippocampus, in contrast to only 1.7-fold increase observed in the aged hippocampus after similar SE.

Overall, it is clear that SE during old age does not enhance hippocampal neurogenesis. Lack of increase in neurogenesis also accounts for greatly reduced number of ectopic granule cells in the dentate hilus of the aged hippocampus after SE. Unchanged neurogenesis following seizures in the DG during old age is consistent with aging studies in hippocampal injury models. These include minimal increase in DG neurogenesis observed after stroke in aged animals (Jin et al., 2004; Darsalia et al., 2005), and an unchanged neurogenesis seen after an

intracerebroventricular KA-induced hippocampal injury in middle-aged and aged animals (Hattiangady et al., 2008). Lack of significant increase in DG neurogenesis after seizures, stroke or hippocampal injury during old age is also consistent with diminished overall injury-induced plasticity in the hippocampus with age (Schauwecker et al., 1995; Woods et al., 1998; Shetty and Turner, 1999; Maughan et al., 2000; Abdel-Rahman et al., 2004; Shetty et al., 2004).

## Possible Reasons for Unchanged Neurogenesis After Seizures During Old Age

Because unchanged neurogenesis after seizures in the aged hippocampus was associated with considerably increased production of new cells in the SGZ-GCL, it is unlikely that the plasticity of NSCs to acute seizures alters with aging. On the contrary, it appears that the proliferation of NSCs in response to seizures increases with aging, as the overall production of new cells is 5.9-folds greater than age-matched controls after SE in the aged hippocampus, in comparison to only 2.6-fold increases in the production of new cells observed after SE in the young adult hippocampus. Thus, it appears that seizures adequately stimulate NSCs in the aged hippocampus to produce more cells. From this perspective, the underlying cause of unchanged neurogenesis after SE in the aged hippocampus points to greatly diminished neuronal fate-choice decision of newly born cells (only 9% of all new cells) in contrast to 76% of neuronal differentiation observed in the age-matched intact control hippocampus.

The above finding is however in contrast to the results of our recent study using an intracerebroventricular KA model of hippocampal injury in aged rats (Hattiangady et al., 2008). In this model, unchanged neurogenesis was associated with diminished production of new cells from NSCs in the SGZ-GCL but neuronal fate-choice of newly born cells was unaltered from control levels, suggesting alterations in NSC plasticity to hippocampal injury with aging. The precise reasons underlying the discrepancy between these two models are not clear. However, one may argue that seizure-induced increases in the production of new cells in the SGZ-GCL of the aged hippocampus is not a result of increased proliferation of NSCs but rather due to increased division of glia (including microglia) in response to seizures and seizure-induced injury. Because phenotypic analyses of newly born cells was performed with only neuronal markers in this study, additional experiments in transgenic animals exhibiting specific labels for NSCs and/or different types of glia will however be needed in future to address the origin of newly born cells in the SGZ-GCL of the aged hippocampus after SE. Alternatively, it is possible that seizures during old age stimulate NSCs to produce new cells but dramatically alter the microenvironment that supports the neuronal differentiation of newly born cells. A number of studies demonstrate that seizure or injury in young adult animals is associated with enhanced concentration of neurotrophic factors that have the ability to stimulate the production of new neurons from NSCs. These comprise BDNF, nerve growth factor (NGF), FGF-2, epidermal growth factor (EGF) and VEGF (Lowenstein et al., 1993; Shetty et al., 2003, 2004; Hagihara et al., 2005). Of these BDNF and FGF-2 may be particularly important for neuronal differentiation of newly born cells from NSCs (Shetty and Turner, 1998; Shetty, 2004). In light of a previous study showing that following KA-induced injury, the aged hippocampus contains 52% less BDNF than the young adult hippocampus (Shetty et al., 2004), it is possible that SE during old age causes severe reductions in the concentration of neuronal differentiation factors in the DG. Furthermore, a study by Deisseroth et al. (2004) demonstrates that increased excitatory stimuli acts directly on adult hippocampal NSCs and influences the production of new neurons. Based on this, one may argue that intraperitoneal KA administration leads to reduced excitatory activity in the aged hippocampus in comparison to the young adult hippocampus. However, both frequency and duration of individual seizures were comparable during the four hours of SE between young adult and aged animals in this study.

Additionally, both young adult and aged animals in this study exhibited similar hippocampal injury after SE. In this context, the results of a previous study on seizure analyses in aged rats are of interest (Darbin et al., 2004). In this study, while both young and aged rats exhibited an increase in the EEG power following the KA treatment, visual inspection and spectral analysis revealed a reduction of the faster frequencies in the EEGs of aged animals despite a shorter latency to stage V seizures in comparison to young rats (Darbin et al., 2004). It is plausible that this altered EEG activity during acute seizures in aged animals inadequately stimulates the differentiation of the progeny of NSCs into neurons in the aged hippocampus and contributes to an unaltered DG neurogenesis following SE. Additional studies are needed in future to address the above possibilities.

### Potential Implications of Unchanged Neurogenesis Following SE During Old Age

As the likelihood for epilepsy in humans rises with aging (Eisenschenk and Gilmore, 1999; LaRoche and Helmers, 2003), it is of interest to understand the role of seizure induced neurogenesis on chronic epilepsy development and cognitive impairments during aging. There is an ongoing controversy whether seizure-induced increases in DG neurogenesis observed in young adult animals are beneficial or detrimental to hippocampal function. Particularly, the question is whether the abnormally increased neurogenesis after SE (with integration of ectopically migrated new granule cells in the dentate hilus) contributes to the development of spontaneous seizures during the chronic phase of epilepsy. A positive link between abnormal neurogenesis and chronic epilepsy development is implied through following findings. These include an increased excitatory afferent input to ectopic granule cells in the dentate hilus (Dashtipour et al., 2001; Pierce et al., 2005), synchronization of spontaneous bursts of action potentials and the spontaneous discharges from ectopic granule cells with CA3 pyramidal cell population discharges (Scharfman et al., 2000), c-fos expression in ectopic granule cells after spontaneous seizures (Scharfman et al., 2001), a direct relationship between the frequency of behavioral seizures and the size of the ectopic granule cell population in the dentate hilus (McCloskey et al., 2006) and reduced frequency of chronic seizures after inhibition of SE-induced neurogenesis via intracerebroventricular infusions of the mitotic inhibitor cytosine-β-D-arabinofuranoside (Jung et al., 2004). Another study however contradicts the potential link between abnormal neurogenesis and chronic epilepsy development. In the amygdala kindling model, ablation of polysialated neural cell adhesion molecule (PSA-NCAM), a molecule that is expressed robustly in immature newly born neurons, does not affect the generation of a hyperexcitable kindled network or associated behavioral changes (Pekcec et al., 2007). Overall, while the available studies do not support any cause-effect relationship between abnormal neurogenesis and chronic epilepsy development after SE, indirect observations support the hypothesis that abnormal neurogenesis contributes to chronic epilepsy. From this viewpoint, the finding that SE in aged rats fails to increase the overall DG neurogenesis and ectopic new granule cells in the dentate hilus may be beneficial for reducing the incidence or intensity of chronic epilepsy after SE during old age. Additional studies are however needed in future to ascertain the impact of minimal ectopic granule cells observed in aged animals after SE on the development of chronic epilepsy.

On the other hand, a study using electrical stimulation model of SE suggests that new granule cells that are born and integrated into the GCL after SE behave differently than new granule cells that are born in the normal DG (Jakubs et al., 2006). The former study demonstrates that granule cells born after SE integrate functionally in such a way that they exhibit reduced excitability, suggesting that newly added granule cells after SE strive to mitigate SE-induced hyperexcitability (Jakubs et al., 2006). Although it is not clear whether the above features of new granule cells are specific to the model studied or applicable to all SE models of epilepsy, this study implies that new granule cells that incorporate into the

GCL after SE are beneficial for dampening DG hyperexcitability. If this turns out to be true for all models of epilepsy, the observation that SE in aged rats fails to increase the overall DG neurogenesis is disadvantageous for minimizing the incidence or intensity of chronic epilepsy after SE during old age. Moreover, in light of the finding that ~14% of newly born neurons in the DG of adult rats differentiate into the inhibitory GABA-ergic basket cells (Liu et al., 2003), it is possible that unaltered DG neurogenesis in the aged hippocampus after SE contributes to an increased susceptibility of the DG to secondary seizures through minimal addition of new GABA-ergic interneurons. Furthermore, many studies show that the spatial memory performance in the aged rats predicts the level of dentate neurogenesis (van Praag et al., 2002; Drapeau et al., 2003, 2007; Bruel-Jungerman et al., 2005; Aimone et al., 2006; Siwak-Tapp et al., 2007), newly formed neurons in the DG get incorporated into learning and memory circuits (Zhao et al., 2006; Ge et al., 2007; Toni et al., 2007), and DG neurogenesis is important for mood (Santarelli et al., 2003). Since the aged hippocampus fails to increase the production of new neurons after SE, it is possible that cognitive impairments and depression related to SE are greater during old age. To understand the impact of unchanged DG neurogenesis after SE during old age, apt interventional strategies that are efficacious for enhancing DG neurogenesis after SE in the aged hippocampus are needed. With such approaches, it may be possible to understand the impact of unchanged DG neurogenesis after SE on hippocampal hyperexcitability, and impairments in learning, memory and mood during old age.

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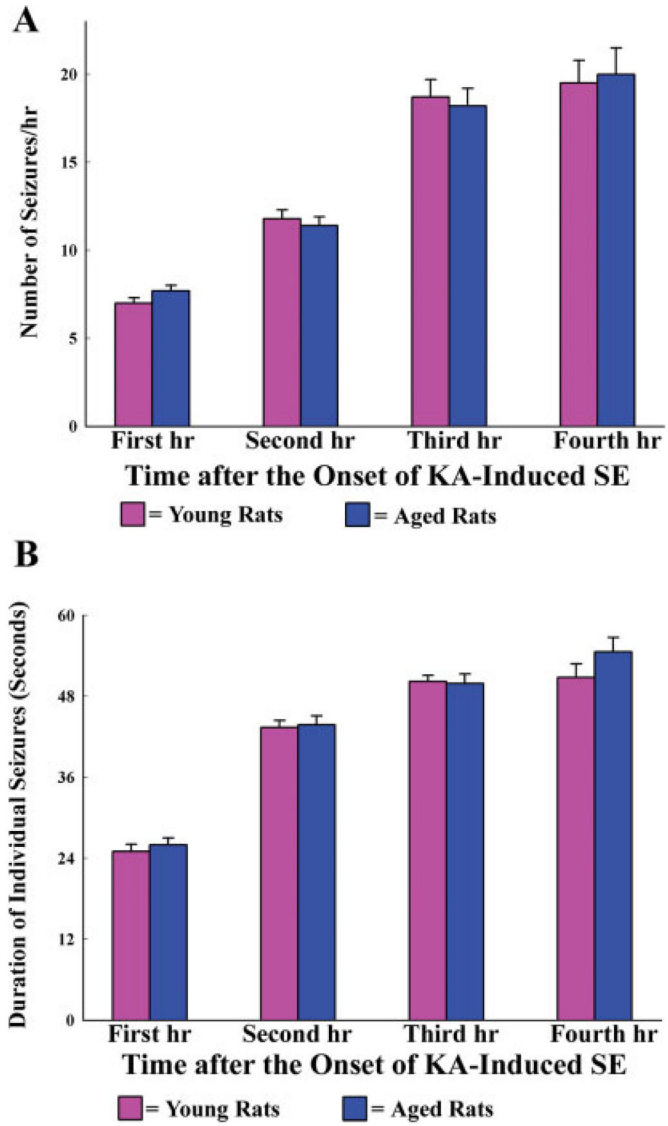


**FIGURE 1.**

Comparison of the number of stages III–V seizures (A) and duration of individual seizures (B) during the 4 h of kainic acid (KA) induced status epilepticus (SE) between young adult and aged animals. Note that both parameters of KA-induced seizures are comparable between the two age groups during SE. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

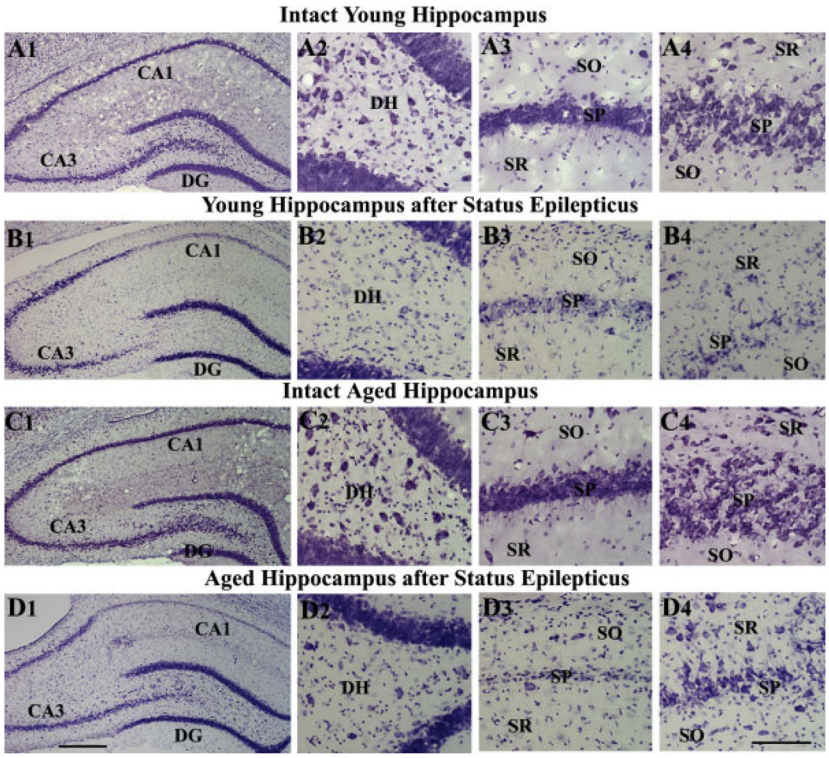


**FIGURE 2.**

Pattern of hippocampal neurodegeneration in young adult and aged animals at 13 days after kainic acid induced status epilepticus (SE), visualized by Nissl staining. Examples show hippocampal cytoarchitecture in a naïve young adult rat (A1), a young adult rat that underwent SE (B1), a naïve aged rat (C1) and an aged rat that underwent SE (D1). Figures A2–A4, B2–B4, C2–C4, and D2–D4 illustrate magnified views of the dentate hilus (A2, B2, C2, D2), the CA1 subfield (A3, B3, C3, D3) and CA3 subfield (A4, B4, C4, D4) from figures A1, B1, C1, D1. Note that considerable neurodegeneration occurs in different regions of the hippocampus in both young adult (B1–B4) and aged (D1–D4) rats after SE. Scale bar, A1, B1, C1, D1 = 400 μm; A2–D4 = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

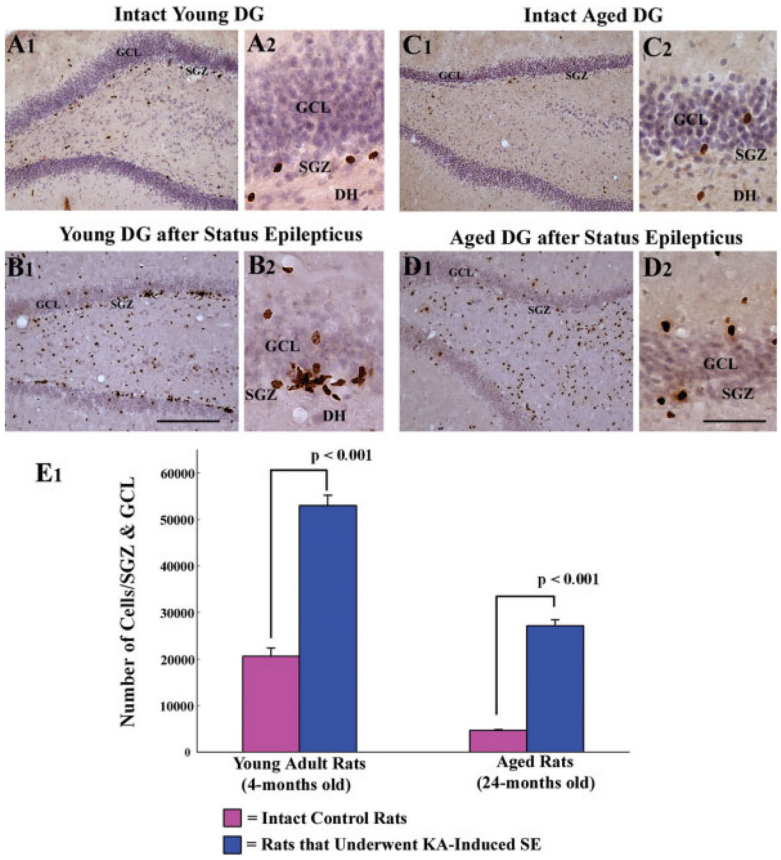


**FIGURE 3.**

Distribution of newly generated cells in the DG at 24 h after 12 daily injections of 5′-bromodeoxyuridine (BrdU) in different groups, visualized with BrdU immunostaining and hematoxylin counterstaining. The groups include the DG of naïve young adult (A1, A2) and aged (C1, C2) rats, and the DG of young adult (B1, B2) and aged (D1, D2) rats that underwent SE. A2, B2, C2, D2 are magnified views of regions from A1, B1, C1, and D1. Note that the density of newly born cells in the subgranular zone-granule cell layer (SGZ-GCL) increases after SE in both young adult rat (B1, B2) and aged rat (D1, D2), in comparison to respective age-matched naïve rats (A1 & A2 and C1 & C2). Also note that the dentate hilus (DH) and CA3c sub region show increased density of BrdU immunopositive cells in rats that underwent SE regardless of the age (B1, D1). The bar chart in E1 compares the absolute numbers of newly born cells added over a period of 12 days to the SGZ-GCL in different groups of rats. Note that, SE considerably increases the numbers of newly born cells in the SGZ-GCL of both young adult and aged rats. Scale bar, A1, B1, C1 D1 = 200 μm; A2, B2, C2, D2 = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

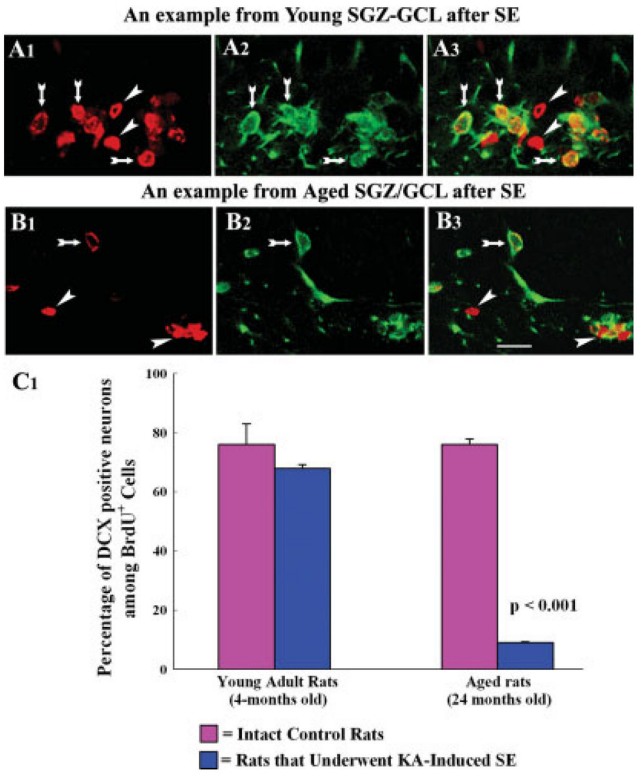


**FIGURE 4.**

Neuronal differentiation of newly born cells in the subgranular zone-granule cell layer (SGZ-GCL) of young adult (A1–A3) and aged (B1–B3) rats that underwent status epilepticus (SE). Analyses was done at 24 h after the last of twelve daily injections of 5′-bromodeoxyuridine (BrdU) and visualized through BrdU and doublecortin (DCX) dual immunofluorescence and confocal microscopy. Note that, a majority of newly born ($BrdU^+$) cells (red nuclei) are positive for DCX (green fluorescence in the cytoplasm of the soma and dendrites) in the young adult group (A1–A3). Arrows indicate some of the newly born cells that differentiate into $DCX^+$ neurons whereas arrowheads denote newly born cells that lack DCX expression. Figure C1 shows that percentages of $BrdU^+$ cells that are positive for DCX in different groups. Note that percentages are comparable between naïve rats and rats that underwent SE in the young adult group. In contrast, in the aged group, SE greatly diminishes the neuronal differentiation of newly born cells. Scale bar, A1–B3 = 20 μm.

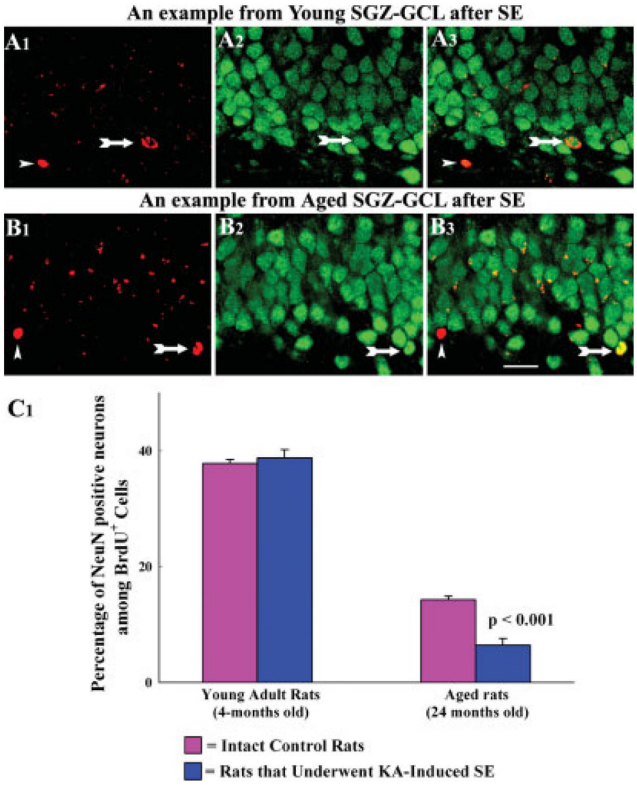


**FIGURE 5.**

Examples of newly added cells that differentiated into neuron-specific nuclear antigen (NeuN) positive mature neurons in the subgranular zone-granule cell layer (SGZ-GCL) of young adult (A1–A3) and aged (B1–B3) rats that underwent status epilepticus (SE). The analysis was done at 24 h after the last of twelve daily injections of 5′-bromodoxyuridine (BrdU) through BrdU and NeuN dual immunofluorescence and confocal microscopy. Arrows indicate some of the newly born cells that differentiate into $NeuN^+$ neurons whereas arrowheads denote newly born cells that lack NeuN expression. Scale bar, 20 μm. The bar chart in C1 demonstrates that 38–39% of new cells differentiate into $NeuN^+$ positive neurons in both naïve young adult rats and young adult rats that underwent SE. On the other hand, in naïve aged rats, only 14% of new cells differentiate into $NeuN^+$ positive neurons and this percentage decreases to 6% after SE.

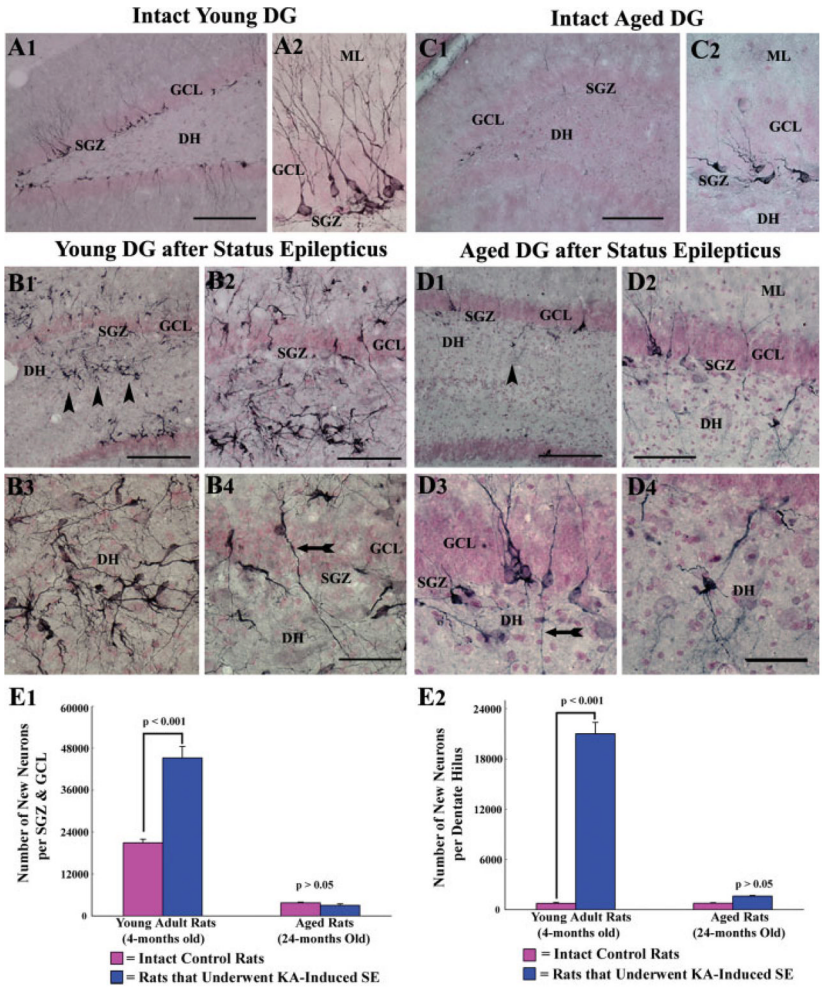


**FIGURE 6.**

Distribution of newly born neurons in the subgranular zone-granule cell layer (SGZ-GCL) of different groups, visualized through doublecortin (DCX) immunostaining. The groups include DG of naïve young adult (A1, A2) and aged (C1, C2) rats, and the DG of young adult (B1, B2) and aged (D1, D2) rats at 13 days after status epilepticus (SE). A2, B2, C2, and D2 are magnified views of regions from A1, B1, C1, and D1. Note that the density of newly born neurons increases in the young adult rat after SE (B1, B2), in comparison to age-matched naïve rat (A1, A2). In contrast, the density of newly born cells in these regions are unchanged in the aged (D1, D2) rat after similar SE, in comparison to the naïve aged rat (C1, C2). Furthermore, in the young adult rat that underwent SE, there is an increased density of ectopically migrated newly born neurons in the dentate hilus (indicated by arrowheads in B1 and magnified views in B2 and B3) and molecular layer (B1, B2). In contrast, in the aged rat that underwent SE, only a few new neurons exhibit ectopic migration into the dentate hilus (arrowhead in D1 and magnified views in D2 and D4). D3 shows some newly born neurons with long basal dendrites (arrows). Scale bars, A1, B1, C1, D1 = 200 μm; B2 and D2 = 100 μm A2, B3, B4, C2, D3, D4 = 50 μm. The bar charts in E1 and E2 compare the absolute numbers of newly born neurons added over a period of 12 days to the subgranular zone-granule cell layer (SGZ-GCL) (E1) and the dentate hilus (E2) between different groups. Note that, SE considerably increases the numbers of newly born cells in both of these regions in young adult rats ($P < 0.001$) but not in aged rats ($P > 0.05$).

[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]