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Decreased Neuronal Differentiation of Newly Generated Cells Underlies Reduced Hippocampal Neurogenesis in Chronic Temporal Lobe Epilepsy

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Abstract

Hippocampal neurogenesis declines substantially in chronic temporal lobe epilepsy (TLE). However, it is unclear whether this decline is linked to altered production of new cells and/or diminished survival and neuronal fate-choice decision of newly born cells. We quantified different components of hippocampal neurogenesis in rats exhibiting chronic TLE. Through intraperitoneal administration of 5'-bromodeoxyuridine (BrdU) for 12 days, we measured numbers of newly born cells in the subgranular zone-granule cell layer (SGZ-GCL) at 24 hours and 2.5 months post-BrdU administration. Furthermore, the differentiation of newly added cells into neurons and glia was quantified via dual immunofluorescence for BrdU and various markers of neurons and glia. Addition of new cells to the SGZ-GCL over 12 days was comparable between the chronically epileptic hippocampus and the age-matched intact hippocampus. Furthermore, comparison of BrdU+ cells measured at 24 hours and 2.5 months post-BrdU administration revealed similar survival of newly born cells between the two groups. However, only 4-5% of newly born cells (i.e. BrdU+ cells) differentiated into neurons in the chronically epileptic hippocampus, in comparison to 73-80% of such cells exhibiting neuronal differentiation in the intact hippocampus. Moreover, differentiation of newly born cells into S-100 β + astrocytes or NG2+ oligodendrocyte progenitors increased to ~79% in the chronically epileptic hippocampus from ~25% observed in the intact hippocampus. Interestingly, the extent of proliferation of astrocytes and microglia (identified through Ki-67 & S-100 β and Ki-67 & OX-42 dual immunofluorescence) in the SGZ-GCL was similar between the chronically epileptic hippocampus and the age-matched intact hippocampus, implying that the proliferation of neural stem/progenitor cells in the SGZ-GCL of the chronically epileptic hippocampus was not obscured by an increased division of glia. Thus, severely diminished DG neurogenesis in chronic TLE is not associated with either decreased production of new cells or reduced survival of newly born cells in the SGZ-GCL. Rather, it is linked to a dramatic decline in the neuronal fate-choice decision of newly generated cells. Overall, the differentiation of newly born cells turns mainly into glia with chronic TLE from predominantly neuronal differentiation seen in control conditions.

Keywords

adult neurogenesis; dentate neurogenesis; depression; granule cells; learning and memory; neural stem cells; spontaneous seizures; stem cell proliferation; stem cell differentiation; temporal lobe epilepsy

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Introduction

Over 50 million people suffer from epilepsy in the world and ~40% of patients exhibiting epilepsy have chronic temporal lobe epilepsy (TLE). A progressive expansion of complex partial seizures arising from the limbic system regions such as the hippocampus is the characteristic feature of TLE (French et al., 1993; Engel et al., 2003). Furthermore, most TLE patients also display learning and memory impairments and depression (Devinsky, 2004; Helmstaedter et al., 2004). While changes due to TLE are apparent in multiple brain regions, the most conspicuous changes appear to be in the hippocampus based on the examination of brain tissues from TLE patients (Sutula et al., 1989; French et al., 1993). Animal prototypes of TLE also exhibit neurodegeneration in the hippocampus as well as several other brain regions but the extent of neurodegeneration varies in different models (Dalby and Mody, 2001; Buckmaster et al., 2002; Brandt et al., 2004; Rao et al., 2006a; Curia et al., 2008). In the hippocampus, significant loss of neurons is seen in the CA1 and CA3 pyramidal cell layer and the dentate hilus (Rao et al., 2006a). Furthermore, while there is no consensus regarding the extent of loss of hippocampal gamma-aminobutyric acid positive (GABA-ergic) interneurons in TLE, some prototypes of TLE exhibit considerable decline in their numbers (Sloviter, 1987; Franck et al., 1988; Shetty and Turner, 2000; 2001; but see Sloviter et al., 2003). Moreover, though controversial, a reduced functional inhibition in the hippocampus is another feature observed in some models of TLE, which is likely due to reduced afferent excitatory input onto interneurons (Cornish and Wheal, 1989; Dudek and Sutula, 2007; however see, Bernard et al., 1998). The above changes are associated with other morphological alterations that are proposed to be epileptogenic, which include the abnormal sprouting and synaptic reorganization of dentate granule cell, entorhinal and CA3 axons (Sutula et al., 1989; Tauck and Nadler, 1985; Shetty, 2002; Shetty et al., 2003, 2005; Siddiqui and Joseph, 2005; Wozny et al., 2005; but see, Longo and Mello, 1998; Williams et al., 2002; Sloviter et al., 2006).

Recent studies in animal models have suggested that altered dentate gyrus (DG) neurogenesis is an additional pathophysiology likely contributing to some of the deficits such as learning and memory impairments and depression observed in TLE. Interestingly, changes in DG neurogenesis are distinct between the early and later phases of the disease in animal models of TLE. The early phase after the initial precipitating injury (IPI) such as status epilepticus (SE) or hippocampal injury is characterized by an increased DG neurogenesis and abnormal migration of a substantial fraction of newly generated granule cells into the dentate hilus (Gray and Sundstrom, 1998; Parent et al., 1997, 2006; Scharfman et al., 2000, 2002, 2003; Gong et al., 2007; Scharfman and Gray, 2007; Kuruba et al., 2009). In contrast, the chronic phase of TLE exhibits substantially declined DG neurogenesis (Hattiangady et al., 2004; Hattiangady and Shetty, 2008a), and is associated with spontaneous recurrent motor seizures (SRMS), learning and memory impairments and depression (Letty et al., 1995; Schwarcz and Witter, 2002; Rao et al., 2006a, 2007). Because of the perceived functions of DG neurogenesis concerning learning, memory and mood (Shors et al., 2001; van Praag et al., 2002; Drapeau et al., 2003; Santarelli et al., 2003; Aimone et al., 2006; Sahay and Hen, 2007; Dupret et al., 2008; Imayoshi et al., 2008), it is plausible that decreased DG neurogenesis during chronic epilepsy contributes to impairments in these functions. Therefore, comprehending the mechanisms underlying decreased DG neurogenesis during chronic epilepsy will be important for developing strategies that improve DG neurogenesis in chronic TLE.

It is currently unclear whether the decreased DG neurogenesis in chronic TLE is linked to altered production of new cells and/or diminished survival and neuronal fate-choice decision of newly born cells. To address these issues, we rigorously quantified different components of DG neurogenesis in male Fischer 344 (F344) rats exhibiting chronic TLE at 6-months after kainic acid (KA) induced SE. We chose 6-months post-SE time-point for analyses of neurogenesis in this study because our pilot studies in chronically epileptic animals have

indicated that the frequency of SRMS remains stable after this time-point for at least until one-year post-SE. To determine the production and survival of newly born cells in the subgranular zone-granule cell layer (SGZ-GCL) of the DG, we measured the numbers of newly born cells in these regions at 24 hours and 2.5 months after daily administration of 5'-bromodeoxyuridine (BrdU) for 12 days in rats exhibiting chronic TLE. To ascertain the neuronal fate-choice decision of newly born cells, we performed BrdU & doublecortin (DCX), BrdU & neuron specific nuclear antigen (NeuN), and BrdU & β -III tubulin (TuJ-1) dual immunofluorescence and confocal microscopic analyses. To elucidate the identity of other newly born cells in the SGZ-GCL, we also performed phenotypic analyses of BrdU+ cells with markers of glia and immature neuronal markers. These comprised analyses of cells positive for mature astrocytes expressing S-100 β , oligodendrocyte progenitors positive for NG2, and immature neurons (with doublecortin and TuJ-1 antibodies). Additionally, we examined the proliferation of glial cells such as astrocytes and microglia in the SGZ-GCL via dual immunofluorescence and confocal microscopic analyses of cells positive for Ki-67 (an endogenous marker of proliferating cells) and S-100 β (a marker of astrocytes), and Ki-67 and OX-42 (a marker of both resting and activated microglia).

Materials and Methods

Animals and kainic acid induced status epilepticus

Young adult (5-months old) F344 rats purchased from Harlan Sprague-Dawley (Indianapolis, IN) were used in this study. All experiments were carried out in accordance with the NIH guide for the care and use of laboratory animals (NIH Publications No. 80-23), and all protocols employed in this study were approved by the Duke University Institutional Animal Care and Use Committee and animal studies subcommittee of the Durham Veterans Affairs Medical Center. The methodology for induction of SE and chronic epilepsy in F344 rats was adapted from the procedure developed earlier by Hellier et al (Hellier et al., 1998) for Sprague-Dawley rats, and the types of seizures emerging after KA administration were scored as per the modified Racine's scale (Hellier et al., 1998). In all rats, SE was induced through graded intraperitoneal injections of KA (3.0 mg/Kg b.w./hr). Because majority of rats (>90%) exhibited greater than 10 stages IV-V seizures during the first hour after the 3rd KA injection, the 4th KA injection was reduced to 1.5 mg/Kg b.w. Thus, each rat received a total KA dose of 10.5 mg/Kg b.w., which is consistent with our previous study (Rao et al., 2006a). 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). Only the animals receiving a total KA dose of 10.5 mg/Kg b.w. and exhibiting >10 stages IV-V seizures during the 3-hr observation after the onset of the SE were included in this study. Stages III-V seizures subsided gradually thereafter and were not apparent at 6 hours after SE. Rats were given moistened rat chow and subcutaneous injections of lactated Ringer's solution (10 ml/day) for 4 days after SE. Animals were housed individually in an environmentally controlled room (~23°C) thereafter with a 12:12-hour light-dark cycle, and were given food and water ad libitum.

Analyses of chronic epilepsy after KA-induced status epilepticus

From the beginning of 3rd month after SE, the frequency of SRMS in all KA-treated rats were assessed for a total duration of three months (i.e. during the 3rd, 4th and 5th months after SE). The scoring of numbers of SRMS was done every week for 8 hours (4 hrs per session, two sessions per week, 32 hrs/month) and the average frequency of seizures per hr was then calculated for every month. The scoring of SRMS utilized a modified Racine's scale (Hellier et al., 1998). For analyses of DG neurogenesis in chronic epilepsy using BrdU injections, rats having similar seizure frequency (ranging from 2.7-3.3 seizures/hr) were chosen at 6 months post-SE (Fig. 1).

Analyses of newly born cells with BrdU administration

Both age-matched intact rats (control group, n=10) and rats exhibiting chronic epilepsy at 6 months post-SE (chronic epilepsy group, n=10) received daily intraperitoneal injections of BrdU for 12 consecutive days at a dose of 100 mg/kg b.w. (Sigma, St Louis, MO). Subsets of BrdU treated rats from both groups were analyzed at 24 hours (n=5/group) and 2.5 months (n=5/group) after the last of 12 BrdU injections (Fig. 1). Rats were anesthetized with halothane and perfused with 4% paraformaldehyde and brains collected for histological analyses of newly born cells and neurons that are added to the SGZ and GCL of the DG. The brains were post-fixed in 4% paraformaldehyde for 16 hours 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.

Immunohistochemistry for visualization of BrdU⁺ cells

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), using avidin-biotin complex method described in our earlier reports (Hattiangady et al., 2004; Rao and Shetty, 2004). The visualization of the peroxidase reaction was done using diaminobenzidine as the chromogen. The immunostained sections were mounted on gelatin-coated slides, air-dried, counter-stained with hematoxylin, dehydrated, cleared and cover slipped.

Measurement of the total number of BrdU⁺ cells in the dentate SGZ and GCL

Cells immunopositive for BrdU 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 each rat belonging to the two groups killed at 24 hours (n = 5/group) and 2.5 months (n = 5/group) after the last of twelve daily BrdU injections. Counting of cells utilized the StereoInvestigator system (Microbrightfield Inc., Williston, VT) interfaced through a Nikon E600 microscope equipped with a color digital video camera (Optronics Inc., Muskogee, OK). Using a 100X oil immersion lens, the BrdU⁺ cells were counted from 60-400 randomly and systematically selected frames (each measuring 40 × 40 μm, 0.0016 mm² area) in every 15th section. The detailed methodology employed for counting is described in our previous reports (Rao and Shetty, 2004; Rao et al., 2006b). In brief, the contour of the SGZ-GCL regions was marked in every section through the tracing function of the StereoInvestigator. The optical fractionator component was then selected and the number and location of counting frames and the counting depth for that section was ascertained via entering parameters such as the grid size, the thickness of the top guard zone (4 μm) and the optical dissector height (8 μm). A computer driven motorized stage then facilitated the section to be analyzed at each of the counting frame locations. All BrdU⁺ cells that were present within the 8μm section depths in each location were counted.

The StereoInvestigator program then calculated the total number of BrdU⁺ cells per SGZ-GCL by utilizing the optical fractionator formula, $N = 1/ssf.1/asf.1/hsf.EQ^-$. The abbreviation ssf represents the section sampling fraction, which was 15 in this study as every 15th section was sampled; asf symbolizes the area sampling fraction, which is calculated by dividing the area sampled with the total area of the SGZ-GCL (i.e. the sum of SGZ-GCL areas sampled in every 15th section); hsf stands for the height sampling fraction, which is calculated by dividing the height sampled (i.e. 8 μm in this study) with the section thickness at the time of analysis (i.e. 15-20 μm); EQ⁻ denotes the total count of particles sampled for the entire DG.

Investigation of DCX+ and TuJ-1+ cells among newly born cells

To assess the percentages of BrdU⁺ cells that express the immature neuronal markers DCX or TuJ-1, representative sections from rats belonging to both control and epileptic groups were processed for BrdU & DCX or BrdU & TuJ-1 dual immunofluorescence staining using sequential immunofluorescence procedures. For these, the sections were first processed for DCX or TuJ-1 immunofluorescence using 10% normal horse serum treatment for 30 minutes and an overnight incubation with goat anti-DCX (1:250, Santa Cruz) or mouse anti-TuJ-1 (1:1000, Covance) primary antibody. The sections were washed in TBS, incubated for 1 hour in an appropriate secondary antibody (biotinylated horse anti-goat IgG [1:200, Vector] for DCX labeling, and biotinylated horse anti-mouse IgG [1:200, Vector] for TuJ-1 labeling), and treated with streptavidin fluorescein (1:100, Molecular Probes) for 1 hour. This procedure gave green fluorescence to soma and dendrites of cells expressing DCX or TuJ-1. Then, the sections were washed thoroughly and processed for BrdU immunofluorescence, which comprised BrdU pre-incubation treatments (Rao et al., 2006b), blocking with 10% normal goat serum, incubating overnight in rat anti-BrdU (1:50, Accurate Chemicals), and treating with goat anti-rat IgG tagged with Alexa Fluor 594 (1:200, Vector) for an hour. Following a thorough rinse in TBS, the sections were cover slipped with slow fade/anti-fade mounting medium (Molecular Probes). This procedure gave red fluorescence to nuclei of cells expressing BrdU, which facilitated identification of cells expressing BrdU and DCX or BrdU and TuJ-1 using confocal laser scanning microscope (LSM 510). The fractions of BrdU⁺ cells that express DCX or TuJ-1 were then quantified by examination of individual BrdU⁺ cells at 400X. Approximately 50 BrdU⁺ cells from 4 sections were examined in each animal (n=4/group). For this, one-micrometer thick optical Z-sections were sampled from different regions of the SGZ-GCL and the images were analyzed using LSM image browser.

Analyses of mature NeuN⁺ neurons among newly born cells

To measure the fractions of BrdU⁺ cells that express the mature neuronal marker NeuN in tissues collected at 2.5 months after BrdU injections, representative sections from rats belonging to both control and epileptic groups were processed for BrdU and NeuN dual immunofluorescence staining. The sections were first processed for various BrdU pre-incubation treatments (Rao et al., 2006b), washed in Tris-buffered saline (TBS), blocked for 30 minutes in a solution containing of 5% normal goat serum and 5% normal rabbit serum in TBS, incubated for 24 hrs in a cocktail antibody solution containing rat anti-BrdU (1:50, Accurate Chemicals) and mouse anti-NeuN (1:1000, Chemicon) and washed in TBS. The sections were then treated for 1 hour with a mixture of goat anti-mouse IgG tagged with Alexa Fluor 488 (1:200) and biotinylated rabbit anti-rat IgG (1:200, Vector), washed in TBS, incubated in streptavidin Texas red (1:200, Molecular Probes) for 1 hr, rinsed thoroughly in TBS, and cover slipped with slow fade/anti-fade mounting medium (Molecular Probes). Cells that exhibited BrdU and NeuN co-expression were identified using a confocal laser scanning microscope (LSM 510). The percentages of BrdU⁺ cells that co-express NeuN were then estimated by examination of individual BrdU⁺ cells at 400X. Approximately 50 BrdU⁺ cells from 4 sections were examined in each animal (n=4/group). This was accomplished through sampling of one-micrometer thick optical Z-sections from different regions of the SGZ-GCL and analyses of images using LSM image browser.

Characterization of glia among newly born cells

To assess the percentages of BrdU⁺ cells that express the markers of astrocytes or oligodendrocyte progenitors, representative sections from rats belonging to both control and epileptic groups were selected for BrdU & S-100 β or BrdU & NG2 dual immunofluorescence staining. The sections were processed for various BrdU pre-incubation treatments (Rao et al., 2006b), washed in TBS, blocked for 30 minutes in TBS solution containing 5% normal goat

serum and 5% normal rabbit serum, incubated for 24 hrs in an antibody solution containing a mixture of rat anti-BrdU (1:200, SeroTech) and mouse anti S-100 β or mouse anti NG2 (1:1000; Chemicon) and washed in TBS. The sections were then immersed for 1 hour in a mixture of goat anti-mouse IgG tagged with Alexa Fluor 488 (1:200) and biotinylated rabbit anti-rat IgG (1:200, Vector), washed in TBS, incubated in streptavidin Texas red (1:200, Molecular Probes) for 1 hr, rinsed thoroughly in TBS, and cover slipped with slow fade/anti-fade mounting medium (Molecular Probes). Cells that displayed BrdU and S-100 β /NG2 co-expression were identified using a confocal laser scanning microscope (LSM 510). The percentages of BrdU⁺ cells that co-express S-100 β or NG2 were then calculated by examination of individual BrdU⁺ cells at 400X. Approximately 50 BrdU⁺ cells from 4 sections were examined in each animal (n=4/group). This was done via sampling of one-micrometer thick optical Z-sections from different regions of the SGZ-GCL and analyses of images using LSM image browser.

Analyses of the proliferation of astrocytes and microglia in the SGZ-GCL

To examine the extent of changes in the proliferation of astrocytes and microglia in the SGZ-GCL with chronic epilepsy, we performed dual immunofluorescence and confocal microscopic analyses of cells positive for Ki-67 & S-100 β , and Ki-67 & OX-42 using brain sections from both chronically epileptic animals and age-matched intact animals. Representative sections from all animals (n=4/group) were blocked for 30 minutes in PBS solution containing 5% normal horse serum and 5% normal goat serum. Following this, sections were incubated overnight in a cocktail of primary antibodies against Ki-67 (rabbit monoclonal, 1:200, Vector) & S-100 β (mouse monoclonal, 1:1000, Chemicon) or Ki-67 & OX-42 (mouse monoclonal, 1:500, SeroTech), rinsed thoroughly in PBS and treated with a mixture of horse anti-rabbit Alexa Fluor 594 (Molecular Probes; 1:200) and biotinylated goat anti-mouse IgG (Vector Labs; 1:250) for 1 hour. Sections were then washed in PBS, incubated in streptavidin fluorescein solution (Molecular Probes; 1:200) for 1 hour, rinsed again in PBS, and mounted on clean slides using slowfade-antifade mounting medium (Molecular Probes). Using a laser confocal microscope (LSM-510 Carl Zeiss), the sections were examined and fractions of Ki-67 cells expressing S-100 β or OX-42 were analyzed. For this, the presence of S-100 β or OX-42 in Ki-67⁺ cells of the SGZ were individually examined using Z sectioning at 1 μ m intervals. Approximately 50 BrdU⁺ cells from 4 sections were examined in each animal (n=4/group). The optical stacks of 5-8 images were used for determination of dual antigen labeling.

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 (n = 4-5) included per group. The values from different groups of animals were compared using two-tailed, unpaired Student's t-test.

Results

Extent of chronic epilepsy at the time of neurogenesis analyses using BrdU injections

We quantified the frequency of SRMS during the 3rd, 4th and 5th months after SE. The scoring of SRMS episodes was done every week for 8 hours (4 hrs per session, two sessions per week, 32 hrs/month) and the average frequency of seizures per hr was then calculated for every month. The average frequency of SRMS in chronically epileptic rats chosen in this study was 2.4 (Mean $\pm$ S.E.M. = 2.4 ± 0.3) per hr during the 3rd month after SE, 2.6 ± 0.2 /hr during the 4th month after SE, and 3.1 ± 0.2 during the 5th month after SE (Fig. 2). Thus, at the time of analyses of DG neurogenesis (i.e. at 6 months post-SE, all animals exhibited robust chronic epilepsy.

Cytoarchitecture of the hippocampus in epileptic rats

Histological examination of brains of chronically epileptic rats demonstrated neurodegeneration in the hippocampus (Fig. 3) and extrahippocampal regions such as the entorhinal cortex, piriform cortex and amygdala (data not illustrated). The neurodegeneration in the hippocampus was typified by an apparent cell loss in the dentate hilus and considerable thinning of cell layers in the CA1 and CA3 regions (Fig. 3 [B1-B2]). However, a few chronically epileptic rats exhibited relatively greater neurodegeneration with considerable loss of CA1 and CA3 pyramidal cell layers at certain antero-posterior levels (Fig. 3 [C1-C2]). These findings are consistent with our earlier quantitative study of neurodegeneration in chronically epileptic rats (Rao et al., 2006a).

Addition of newly born cells to granule cell layer

Analyses of BrdU+ cells at 24 hours after the last of 12 daily BrdU injections revealed the presence of newly born cells in the SGZ-GCL of both age-matched control rats and chronically epileptic rats (Fig. 4 [A1-B2]). Furthermore, the distribution of newly born cells in the SGZ-GCL appeared similar between the two groups despite an increased density of newly born cells (BrdU+ cells) in the dentate hilus of the chronically epileptic hippocampus (Fig. 4 [A1-B2]). Measurement of BrdU labeled cells using stereology revealed that an average of 4,945 (Mean $\pm$ S.E.M. = $4,945 \pm 517$, $n=5$) cells were added to the SGZ-GCL of the chronically epileptic hippocampus during a 12 day-period (Fig. 4 [C1]). Likewise, in the age-matched intact hippocampus, 5,105 ($5,105 \pm 242$, $n=5$) cells were added to the SGZ-GCL during the same period (Fig. 4 [C1]). Thus, the overall addition of new cells to the SGZ-GCL in chronic epilepsy is similar to that observed in the age-matched intact hippocampus.

Survival of newly born cells

Examination of BrdU+ cells at 2.5 months after the last of twelve daily BrdU injections revealed long-term survival of newly born cells in the SGZ-GCL of both age-matched intact hippocampus and chronically epileptic hippocampus (Fig. 4 [D1-E2]). Measurement of BrdU + cells in the SGZ-GCL of chronically epileptic rats at 2.5 months after the last of twelve daily BrdU injections revealed that the extent of long-term survival of newly born cells in the chronically epileptic hippocampus is comparable to that observed in age-matched intact hippocampus (Fig. 4 [F1]). The overall survival of new cells added over a period of twelve days declined from an average of 4,945 cells (at 24 hours after the last of twelve BrdU injections) to an average of 2,942 cells ($2,942 \pm 164$, $n=5$) at 2.5 months after BrdU injections in the chronically epileptic hippocampus (Fig. 4 [F1]). This pattern of survival was found to be comparable to the decline observed in the age-matched intact hippocampus during the same period (i.e. from an average of 5,105 to an average of 2,785 [$2,785 \pm 108$, $n=5$] cells). Thus, ~59% of cells added over a period of 12 days exhibited long-term survival in the chronically epileptic hippocampus, which is highly similar to the long-term survival of ~55% of cells observed in the age-matched intact control hippocampus (Fig. 4 [F1]). Thus, the microenvironment of the chronically epileptic hippocampus is capable of supporting considerable long-term survival of newly born cells.

Differentiation of newly born cells at 24 hrs after BrdU injections

We analyzed the differentiation of newly born cells in the SGZ-GCL into neurons and glia at 24 hours after twelve daily injections of BrdU using dual immunofluorescence and confocal microscopic analyses in both groups. Newly born cells (i.e. BrdU+ cells) differentiated into DCX+ and TuJ-1+ neurons, S-100 β + astrocytes and NG2+ oligodendrocyte progenitors in both groups. Representative examples of these cells are illustrated in Figure 5 (A1-D1). The overall differentiation of newly born cells into DCX+ neurons was $3.6 \pm 0.8\%$ in the chronically epileptic hippocampus and $72.6 \pm 1.7\%$ in the age-matched intact hippocampus ($p < 0.0001$;

Fig. 5 [E1]). Differentiation of newly born cells into TuJ-1+ neurons at this time-point was $7.6 \pm 2.7\%$ in the chronically epileptic hippocampus and $19.9 \pm 1.0\%$ in the age-matched intact hippocampus ($p < 0.01$; Fig. 5 [E1]). These results suggest a severely impaired neuronal differentiation of newly born cells in the chronically epileptic hippocampus. However, differentiation of newly born cells into S-100 β + astrocytes was found to be increased in the chronically epileptic hippocampus ($36.2 \pm 4.6\%$) in comparison to the age-matched intact hippocampus ($13.1 \pm 4.8\%$; $p < 0.05$; Fig. 5 [E1]). Additionally, differentiation of newly generated cells into NG2+ oligodendrocyte progenitors was enhanced considerably in the chronically epileptic hippocampus ($58.9 \pm 3.2\%$) when compared to their counterparts in the age-matched intact hippocampus ($21 \pm 3.4\%$; $p < 0.001$). Thus, the phenotypic differentiation of newly born cells in the SGZ-GCL of the chronically epileptic hippocampus is very distinct from the phenotypic differentiation of their counterparts in the SGZ-GCL of the age matched intact hippocampus. Overall, with the emergence of chronic TLE, the differentiation of newly born cells turns mainly into glia.

Fraction of neurons among newly generated cells that exhibit long-term survival

Investigation with BrdU-NeuN dual immunofluorescence and confocal microscopic analyses revealed the presence of mature neurons among newly generated cells (i.e. BrdU+ cells) that survived 2.5 months in both groups. Examples of BrdU+ cells with or without NeuN co-expression are illustrated in figure 6. Analyses of the fractions of BrdU+ cells expressing NeuN revealed a dramatically decreased neuronal differentiation of newly born cells in the chronically epileptic hippocampus. Only 4% ($4.1 \pm 0.5\%$) of newly born cells that survived 2.5 months exhibited NeuN expression in the chronically epileptic hippocampus, which is in sharp contrast to NeuN expression in 80% ($80 \pm 1.3\%$) of newly born cells that survived 2.5 months in the age-matched intact hippocampus. These findings are consistent with the results obtained at 24 hrs after BrdU injections with BrdU-DCX immunofluorescence analyses described above. Thus, a dramatically decreased neuronal differentiation of newly born cells observed in the chronically epileptic hippocampus at 24 hrs after BrdU injections is not due to a general delay in the neuronal differentiation of newly born cells in the chronically epileptic hippocampus. To further rule out the possibility of a fraction of newly generated neurons remaining immature in the chronically epileptic hippocampus, we analyzed BrdU+ cells expressing TuJ-1 (Fig. 7 [A1]). This showed that only $4.9 \pm 1.8\%$ of BrdU+ cells expressed TuJ-1, which is comparable to the fraction of BrdU+ cells expressing NeuN described above. Taken together, these results clearly demonstrate that severely diminished DG neurogenesis during chronic epilepsy is a result of dramatic decline in the neuronal fate-choice by the newly generated cells. The results also suggest that the microenvironment of the SGZ-GCL is not conducive for significant neuronal differentiation of newly born cells in chronic epilepsy.

Extrapolation of BrdU-NeuN data with the total number of BrdU positive cells measured at 2.5 months after BrdU injections revealed that ~118 mature neurons (118 ± 6.6) are added to the GCL over a period of 12 days in the chronically epileptic hippocampus. It is noteworthy that the overall addition is 95% less than that observed in the age-matched intact hippocampus where ~2,228 mature neurons ($2,228 \pm 86.4$) are added to the GCL during the same period. These results are consistent with our previous data based on counts of newly born neurons exhibiting the immature neuronal marker doublecortin, where the chronically epileptic hippocampus exhibited 81% less number of newly born neurons than the age-matched control hippocampus (Hattiangady et al., 2004).

Astrocytes and oligodendrocyte progenitors among newly generated cells that exhibit long-term survival

Analyses with BrdU & S-100 β or BrdU & NG2 dual immunofluorescence and confocal microscopic analyses revealed the presence of both mature astrocytes and NG2+

oligodendrocyte progenitors among newly generated cells (i.e. BrdU+ cells) that survived 2.5 months in both groups. Representative examples of BrdU+ cells that express S-100 β or NG2 are illustrated in figure 7 [B1, C1]). Newly born cells that expressed S-100 β + (i.e. astrocytes) were greater in the chronically epileptic hippocampus ($42 \pm 4.8\%$) than in the age-matched intact hippocampus ($12.0 \pm 1.7\%$; $p < 0.01$; Fig. 7 [D1]). Similarly, greater fractions of newly generated cells expressed NG2 (i.e. oligodendrocyte progenitors) in the chronically epileptic hippocampus ($37.4 \pm 7.2\%$) than in the age-matched intact hippocampus ($12.5 \pm 3.4\%$; $p < 0.05$; Fig. 7 [D1]). Thus, newly born cells that survive for prolonged periods in the SGZ-GCL of the chronically epileptic hippocampus mostly become astrocytes or oligodendrocyte progenitors unlike the age-matched intact hippocampus where newly born cells that survive for extended periods in the SGZ-GCL predominantly become neurons.

Proliferation of astrocytes and microglia in the SGZ-GCL

Investigation using dual immunofluorescence for Ki67 & S-100 β , and Ki-67 & OX-42 revealed proliferating astrocytes and microglial cells in the SGZ-GCL of both the age-matched intact hippocampus (Fig. 8 [A1, B1]) and the chronically epileptic hippocampus (Fig. 8 [A2, B2]). In the SGZ-GCL of the intact hippocampus, $21.3 \pm 3.3\%$ of Ki-67+ cells expressed S-100 β and $17.9 \pm 2.5\%$ of Ki-67+ cells expressed OX-42 (Fig. 8 [C1]). Interestingly, these percentages are similar to that observed in the SGZ-GCL of the chronically epileptic hippocampus (Ki-67 & S-100 β , $19.3 \pm 2.5\%$; Ki-67 & OX-42, $21.9 \pm 4.3\%$; Fig. 8 [C1]). Thus, percentages of dividing cells that express S-100 β or OX-42 in the SGZ-GCL are similar between the age-matched intact hippocampus and the chronically epileptic hippocampus. This suggests that chronic TLE does not increase the proliferation of mature astrocytes and microglial cells in the SGZ-GCL, despite increases seen in the proliferation of such cells in other regions of the chronically epileptic hippocampus (data not quantified). These results also imply that the proliferation of neural stem/progenitor cells (NSCs) in the SGZ-GCL of the chronically epileptic hippocampus was not obscured by an increased division of glia in this study.

Discussion

Our earlier study, using counts of DCX+ newly born neurons has demonstrated substantially decreased DG neurogenesis in a rat model of chronic TLE (Hattiangady et al., 2004). This finding was also supported by observations in the hippocampal tissues resected from human TLE patients (Crespel et al., 2005; Fahrner et al., 2007). However, the reasons underlying this decrease were not clear. Through quantitative analyses of production, survival and neuronal differentiation of newly born cells, we now provide novel evidence that severely diminished DG neurogenesis in chronic TLE is mainly due to a dramatic decrease in the neuronal fate-choice decision of newly born cells in the SGZ-GCL.

The observations that substantiate the above finding include the following. First, the numbers of newly born cells added to the SGZ-GCL over a period of 12 days, and numbers of newly born cells that exhibit 2.5 months survival are similar between the chronically epileptic hippocampus and the age-matched intact hippocampus. Second, phenotypic analyses of newly born cells shortly after their birth in the SGZ-GCL reveals that only 4% of newly born cells differentiate into DCX+ immature neurons in the chronically epileptic hippocampus in contrast to 73% of newly generated cells differentiating into DCX+ immature neurons in the age-matched intact hippocampus. Third, among newly born cells that exhibit 2.5 months survival, only 4% express the mature neuronal marker NeuN in the chronically epileptic hippocampus, in comparison to 80% of such cells expressing NeuN in the age-matched intact hippocampus. Fourth, chronic epilepsy considerably increases the differentiation of newly born cells into glia. Fifth, increased glial differentiation of newly born cells observed in the chronically epileptic hippocampus is not due to an increase in the proliferation of mature glia in the SGZ-

GCL of the chronically epileptic hippocampus as the populations of proliferating glia in the SGZ-GCL are comparable between the chronically epileptic hippocampus and the age-matched intact hippocampus. Collectively, the results suggest that chronic TLE impairs neither the addition nor survival of new cells in the SGZ and GCL but interferes considerably with neurogenesis through diminishing neuronal differentiation of newly born cells.

Origin of new cells in the SGZ-GCL of chronically epileptic hippocampus

While similar numbers of newly born cells were found in the SGZ and GCL over a period of 12 days between the chronically epileptic hippocampus and the age-matched intact hippocampus, the precise origin of new cells in the SGZ-GCL of the epileptic hippocampus could not be determined because of multiple BrdU injections over 12 days. Although it is generally presumed that newly born cells in the SGZ-GCL of the normal intact hippocampus represent cells that are mostly generated through proliferation of NSCs and fast cycling transit amplifying cells derived from NSCs (McDonald and Wojtowicz, 2005; Rao et al., 2006b; Hattiangady and Shetty, 2008b), one might argue that most of the newly born cells in the SGZ-GCL of the chronically epileptic hippocampus could represent cells that are derived from proliferation of mature glia such as astrocytes and microglial cells. This is because chronic TLE is generally associated with significant proliferation of astrocytes and microglia in the DG and other regions of the hippocampus (Niquet et al., 1994; Vessal et al., 2005). Furthermore, a study examining the hippocampi from patients with mesial TLE and hippocampal sclerosis also reports considerable gliosis in the hippocampus (Crespel et al., 2005). However, specific analyses of the proliferation of astrocytes and microglia using Ki-67 & S-100 β and Ki-67 & OX-42 dual immunofluorescence and confocal microscopy in this study revealed that chronic epilepsy does not increase the proliferation of mature astrocytes and microglial cells in the SGZ-GCL, while increases in the proliferation of such cells was evident in the dentate hilus, and CA1 and CA3 subfields. Thus, it appears that the unaltered addition of new cells to the SGZ-GCL of the chronically epileptic hippocampus (in comparison to age-matched intact hippocampus) is a result of both continued proliferation of NSCs and other cells in these regions, as observed in the age-matched intact hippocampus.

From the above perspective, it is plausible that the microenvironment of the chronically epileptic hippocampus is adequate for maintaining significant proliferation of NSCs in the SGZ. This scenario is possible because certain factors that are mitogenic to NSCs are considerably up regulated in the chronically epileptic hippocampus. These include the vascular endothelial growth factor (VEGF) and neuropeptide Y (NPY) (Silva et al., 2005; Rao et al., 2006a; Rigau et al., 2007). Because of their ability to induce proliferation of NSCs (Jin et al., 2002; Howell et al., 2005; Sun et al., 2006), it is likely that increased VEGF and NPY levels maintain proliferation of NSCs during chronic epilepsy at levels observed in the age-matched intact hippocampus. The presence of recurrent seizures might be another factor which maintains NSC proliferation in the chronically epileptic hippocampus. Taken together, it is possible that, increased VEGF and NPY levels and persistent spontaneous seizures compensate for the down-regulation of several other NSC mitogenic factors such as the fibroblast growth factor-2 (FGF-2), insulin-like growth factor-1 (IGF-1), and brain-derived neurotrophic factor (BDNF) (Shetty et al., 2003; Wozny et al., 2005), and wingless-type MMTV integration site family (Wnt) protein in chronic epilepsy (Lie et al., 2005; Busceti et al., 2007).

Survival and differentiation of newly born cells in the SGZ-GCL of chronically epileptic hippocampus

The finding that numbers of newly born cells that exhibit 2.5 months survival in the SGZ-GCL are similar between the chronically epileptic hippocampus and the age-matched intact hippocampus suggests that the microenvironment of the chronically epileptic hippocampus is adequate for supporting long-term survival of newly born cells. However, phenotypic analyses

of newly born cells shortly after their birth in the SGZ-GCL demonstrated that only 4% of newly born cells differentiated into DCX+ immature neurons in the chronically epileptic hippocampus in contrast to 73% of newly generated cells differentiating into DCX+ immature neurons in the age-matched intact hippocampus. Because DCX is an immature marker of newly born neurons (Kempermann et al., 2003; Rao and Shetty, 2004), it may be argued that newly born neurons in the chronically epileptic hippocampus lose DCX expression faster than their counterparts in the age-matched intact hippocampus because newly born neurons tend to exhibit faster growth in the presence of seizures (Overstreet-Wadiche et al., 2006). However, analyses of newly born cells with BrdU and TuJ-1 (a relatively mature neuronal marker than DCX) dual immunolabeling at 24 hrs after BrdU injections in this study effectively ruled out this scenario, as only 8% of newly born cells expressed TuJ-1 in the chronically epileptic hippocampus in comparison to 20% of newly born cells expressing TuJ-1 in the age-matched intact hippocampus. Moreover, analyses of the phenotype of newly born cells using glial markers revealed that significantly greater fractions of newly born cells differentiate into S-100 β + astrocytes and NG2+ oligodendrocyte progenitors in the chronically epileptic hippocampus, in comparison to their counterparts in the age-matched intact hippocampus. Furthermore, among newly born cells that exhibited 2.5 months survival, only 4% expressed the mature neuronal marker NeuN in the chronically epileptic hippocampus, in comparison to 80% of such cells expressing NeuN in the age-matched intact hippocampus. Analyses of surviving newly born cells at 2.5 months post BrdU injections using BrdU-TuJ-1 dual immunofluorescence in the chronically epileptic hippocampus also revealed similar low-level of neuronal differentiation (i.e. 5% of all surviving cells).

Characterization of remaining BrdU+ cells further demonstrated that a vast majority of newly added cells that survive 2.5 months (~79% of cells) differentiate into S-100 β + astrocytes or NG2+ oligodendrocyte progenitors in the chronically epileptic hippocampus, in comparison to ~25% of newly added cells differentiating into S-100 β + astrocytes or NG2+ oligodendrocyte progenitors in the age-matched intact hippocampus. Reduced neuronal differentiation of newly born cells in the chronically epileptic hippocampus observed in this study, on the other hand, is consistent with the previous report in a mouse model of hippocampal sclerosis (Kralic et al., 2005). In the latter study, 70-90% of newly born cells in the SGZ of the injured hippocampus differentiated into glia, in contrast to the age-matched intact hippocampus where ~75% of newly born cells differentiated into neurons (Kralic et al., 2005). Thus, the differentiation of newly born cells in the SGZ-GCL of the chronically epileptic hippocampus is very distinct from the phenotypic differentiation of their counterparts in the SGZ-GCL of the aged matched intact hippocampus. Overall, the differentiation of newly born cells turns mainly into glia with chronic TLE from predominantly neuronal differentiation seen in control conditions. The results also underscore that the microenvironment of the DG is not conducive for significant neuronal differentiation of newly born cells during chronic epilepsy. Dramatically reduced neuronal differentiation of newly born cells likely reflects paucity of factors that support neuronal differentiation of newly born cells in the milieu of the chronically epileptic hippocampus. This may include decreased levels of BDNF, IGF-1, FGF-2 and Wnt proteins in the chronically epileptic hippocampus (Shetty et al., 2003; Hattiangady et al., 2004; Busceti et al., 2007). Additionally, loss of reelin expression in the epileptic hippocampus (Heinrich et al., 2006; Gong et al., 2007;) likely contributes to decreased neuronal differentiation because reduced neurogenesis with preferential differentiation of newly born cells into astrocytes was observed in mice lacking reelin (Zhao et al., 2007).

Potential consequences of declined DG neurogenesis in chronic TLE

There may be several consequences of declined DG neurogenesis in chronic TLE. To begin with, a study using an electrical stimulation model of SE suggests that granule cells that are born and integrated into the GCL after SE integrate in such a way that they receive reduced

excitatory synaptic input and exhibit an enhanced inhibitory synaptic drive (Jakubs et al., 2006). From this view point, it may be that decreased addition of new neurons into the GCL reduces the possible spontaneous repair of DG hyperexcitability. Nevertheless, it is of interest to note that the integration of newly born granule cells in the epileptic hippocampus is not always into the GCL, as considerable fractions of newly born neurons migrate abnormally into the dentate hilus or the dentate molecular layer and establish aberrant synaptic connectivity with CA3 pyramidal neurons and afferent axons coming from the entorhinal cortex (Scharfman et al., 2000, 2002, 2003; Scharfman and Gray, 2007). Furthermore, new granule cells that integrate into the SGZ-GCL in epileptic conditions in addition display an increased occurrence of basal dendrites (Ribak et al., 2000; Arisi and Garcia-Cairasco; Jessberger et al., 2007; Walter et al., 2007), a feature known to promote aberrant synaptic reorganization in the epileptic hippocampus. Thus, the overall contribution of decreased DG neurogenesis to the extent and intensity of spontaneous seizures in chronic TLE remains to be elucidated.

The other important consequences of decreased DG neurogenesis in chronic TLE are likely on functions such as learning, memory and mood. Because the maintenance of hippocampal dependent learning and formation of the temporal clusters of long-term episodic memories likely require continuous addition of newly functional granule cells into the DG circuitry (Shors et al., 2002; van Praag et al., 2002; Aimone et al., 2006; Kee et al., 2007; Dupret et al., 2008; Imayoshi et al., 2008), decreased addition of new functional granule cells into the DG in chronic epilepsy likely contributes to hippocampal-dependent learning and memory impairments associated with chronic TLE. Clinical studies reporting that patients with a longer duration of refractory TLE exhibit more severe cognitive impairments supports this possibility (Jokeit and Ebner, 1999; Alessio et al., 2004). However, cause-effect relationship between decreased DG neurogenesis and learning and memory impairments in chronic TLE remains to be established in future studies. Moreover, in view of the suggested role of hippocampal neurogenesis in mediating the behavioral effects of antidepressants (Santarelli et al., 2003; Sahay and Hen, 2007), reduced DG neurogenesis might contribute to the pathophysiology of depression observed in patients with chronic TLE. Thus, it is likely that decreased DG neurogenesis at least partially contributes to learning and memory impairments and depression observed in chronic TLE.

Considering the above, development of strategies that selectively enhance the addition of new neurons into the SGZ-GCL of the chronically epileptic hippocampus (through increased neuronal differentiation of newly born cells) may be beneficial for alleviating learning and memory impairments and depression in chronic TLE patients. In this context, approaches that enhance DG neurogenesis such as grafting of neural stem cells into the hippocampus (Chu et al., 2004; Hattiangady et al., 2007), administration of neurotrophic factors such as FGF-2 and IGF-1 (Lichtenwalner et al., 2001; Jin et al., 2003; Rai et al., 2007), regular physical exercise (Arida et al., 2004; van Praag et al., 2005; Cotman et al., 2007), exposure to enriched environment (Faverjon et al., 2002; Koh et al., 2007) and antidepressant therapy (Sahay and Hen, 2007) might be beneficial for improving cognitive function and mood in chronic TLE. However, it remains to be validated whether the overall effects of increased DG neurogenesis during chronic epilepsy will be beneficial for reducing the frequency and intensity of SRMS because of the suggested role of abnormal DG neurogenesis occurring at early time-points after SE towards the development of chronic epilepsy (Scharfman et al., 2000, 2002, 2003; Scharfman and Gray, 2007). Rigorous long-term investigations are however needed in animal models exhibiting chronic epilepsy to characterize the extent of improvements with the above approaches.

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RESEARCH DESIGN

Figure 1.

Schematic of major experiments performed in this study. Different components of dentate gyrus (DG) neurogenesis were measured in male F344 rats exhibiting chronic TLE at 6-months after kainic acid (KA) induced SE and age-matched control rats. Production and survival of newly born cells in the subgranular zone-granule cell layer (SGZ-GCL) was measured in both groups through stereological counting of 5'-bromodeoxyuridine (BrdU) labeled cells at 24 hours and 2.5 months after twelve injections of BrdU over 12 days. Furthermore, phenotypic characterization of newly born cells was performed using dual immunofluorescence and confocal microscopy for BrdU & doublecortin (DCX), BrdU & beta-III tubulin (TuJ-1), BrdU & neuron specific nuclear antigen (NeuN), BrdU & S-100 β (a marker of astrocytes), BrdU & NG2 (a marker of oligodendrocyte progenitors) at 24 hrs and 2.5 months after BrdU injections. Additionally, the extent of proliferation of mature astrocytes and microglial cells was characterized in the SGZ-GCL using Ki-67 (an endogenous marker of proliferating cells) & S-100 β , and Ki-67 & OX-42 dual immunofluorescence and confocal microscopy.

Time after Status Epilepticus

Figure 2.

Extent of chronic epilepsy in rats chosen for analyses of dentate gyrus (DG) neurogenesis at 3rd, 4th, and 5th months after status epilepticus. Note that, at the time of analyses of DG neurogenesis, all animals display robust chronic epilepsy, characterized by spontaneous recurrent motor seizures (SRMS).

Figure 3.

Comparison of the hippocampal cytoarchitecture between an age-matched intact rat (A1, A2) and chronically epileptic rats (B1-C2). Note that, chronically epileptic rats (B1-C2) exhibit neurodegeneration in the hippocampus. In most chronically epileptic rats, neurodegeneration is mainly typified by loss of neurons in the dentate hilus and thinning of cell layers in the CA1 and CA3 regions (B1-B2). However, some chronically epileptic rats display relatively greater level of neurodegeneration in one of the hippocampi with significant loss of CA1 and CA3 pyramidal cell layers (C1-C2). Scale bar, 400 μ m.

New Cells in the DG at 24 Hours after 12 Daily BrdU Injections

Age-matched Control Rat

Chronically Epileptic Rat

Surviving Newly Born Cells at 2.5 Months after 12 Daily BrdU Injections

Age-matched Control Rat

Chronically Epileptic Rat

= Age-Matched Control Hippocampus
 = Chronically Epileptic Hippocampus

Figure 4.

Left Panel: Addition of newly born cells to the dentate gyrus over a period of 12 days, as examined through 5'-bromodeoxyuridine (BrdU) immunostaining at 24 hours after 12 daily injections of BrdU. A1 and B1 show the distribution of BrdU positive cells in an age-matched control rat (A1) and a chronically epileptic rat (B1). A2 and B2 are magnified views of regions from A1 and B1 demonstrating the morphology and location of BrdU positive cells. The bar chart C1 illustrates that the absolute numbers of new cells added over a period of 12 days are similar between the control and epileptic groups. **Right panel:** Long-term survival of new cells that are added over a period of 12 days, as examined through 5'-bromodeoxyuridine (BrdU) immunostaining at 2.5 months after 12 daily injections of BrdU. The figures D1 and E1

illustrate the distribution of BrdU positive cells in an age-matched control rat (D1) and a chronically epileptic rat (E1). Note that newly added cells survive in the subgranular zone-granule cell layer (SGZ-GCL) for 2.5 months in both groups. D2 and E2 are magnified views of regions from D1 and E1 demonstrating the morphology and location of BrdU positive cells. Note that newly added cells migrate up into the granule cell layer in both groups. The bar chart F1 illustrates that the long-term survival of new cells that are added over 12 days is similar between the control and epileptic groups. Scale bar, A1, B1, D1, E1 = 200 μm ; A2, B2, D2, E2 = 50 μm .

Figure 5.

Differentiation of newly born cells into neurons, astrocytes, and oligodendrocyte progenitors at 24 hrs after twelve daily injections of BrdU in the chronically epileptic hippocampus. Examples of newly born cells that differentiate into doublecortin + (DCX+) immature neuron (A1-A3), TuJ-1+ neuron (B1; indicated by an arrow), S-100β+ astrocyte (C1; denoted by an arrow), and NG2+ oligodendrocyte progenitor (D1; showed by an arrow) in the subgranular zone-granule cell layer (SGZ-GCL) are illustrated. Scale bar, A1-A3 = 5 μm; B1, C1, D1 = 10 μm. The bar chart in E1 compares percentages of newly born cells (i.e. BrdU+ cells) that express DCX, TuJ-1, S-100β or NG2 in the subgranular zone-granule cell layer (SGZ-GCL) between the age-matched intact hippocampus and the chronically epileptic hippocampus. Note that the neuronal differentiation of newly born cells is dramatically decreased but differentiation of newly added cells into S-100β+ or NG2+ glia is considerably enhanced in the chronically epileptic hippocampus.

Figure 6.

Neuronal differentiation of newly born cells in the subgranular zone-granule cell layer (SGZ-GCL) of chronically epileptic hippocampus. The left panel (A1-A3) shows a newly added cell (i.e. BrdU positive cell) that differentiated into neuron-specific nuclear antigen (NeuN) positive neuron. The right panel (B1-B3) shows a newly added cell (i.e. BrdU positive cell) that did not differentiate into neuron-specific nuclear antigen (NeuN) positive neuron. A4 and B4 illustrate orthogonal views of newly added cells (BrdU positive cells) marked in A1 and B1 and demonstrate that the newly added cell in A4 exhibits NeuN co-expression (indicated by orange color) whereas the newly added cell shown in B4 lacks NeuN expression (indicated by only red color). The analysis was done at 2.5-months after twelve daily injections of 5'-bromodeoxyuridine (BrdU) through BrdU and NeuN dual immunofluorescence and confocal microscopy. Analyses reveal that only 4% of newly added cells differentiate into neurons. DH, dentate hilus; ML, molecular layer. Scale bar, A1-A3 and B1-B3, 20 μm ; A4 and B4, 5 μm .

Figure 7.

Phenotype of newly added cells (i.e. BrdU+ cells) in the subgranular zone-granule cell layer (SGZ-GCL) of chronically epileptic hippocampi at 2.5 months after their birth. Examples of cells that differentiated into TuJ-1+ neurons (A1), S-100β+ astrocytes (B1), and NG2+ oligodendrocyte progenitors (C1) are illustrated. Scale bar, 10 μm. The bar chart in D1 compares percentages of newly born cells (i.e. BrdU+ cells) that express S-100β or NG2 in the SGZ-GCL at 2.5 months after BrdU injections between the age-matched intact hippocampus and the chronically epileptic hippocampus. Note that the differentiation of newly added cells into glia is greatly enhanced in the chronically epileptic hippocampus.

Figure 8.

Examples of proliferating astrocytes and microglial cells within the subgranular zone-granule cell layer (SGZ-GCL) of age-matched intact hippocampus (A1, B1) and chronically epileptic hippocampus (A2, B2), visualized through dual immunofluorescence for Ki67 & S-100 β (A1, A2), and Ki-67 & OX-42 (B1, B2). Scale bar, 10 μ m. The bar chart in C1 compares percentages of Ki-67+ cells that express S-100 β or OX-42 in the SGZ-GCL between the age-matched intact hippocampus and the chronically epileptic hippocampus. Note that, the percentages of dividing cells that express S-100 β or OX-42 in the SGZ-GCL are similar between the two groups.