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# Degeneration and Regeneration of GABAergic Interneurons in the Dentate Gyrus of Adult Mice in Experimental Models of Epilepsy

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## SUMMARY

**Aims:** Mounting evidence showed that GABAergic interneurons play an important role in the generation of seizures by regulating excitatory/inhibitory balance in the hippocampus; however, there is a continuous debate regarding the alteration in the number of hippocampal GABAergic interneurons during epileptogenesis. Here, we investigated the degeneration and regeneration of GABAergic interneurons in the dentate gyrus during epileptogenesis using glutamic acid decarboxylase-green fluorescence protein (GAD67-GFP) knock-in mice. **Methods and Results:** Pentylenetetrazol (PTZ)-induced chronic kindling model and lithium–pilocarpine-induced status epilepticus (SE) model were used in this study. We found a progressive loss of GABAergic interneurons in the dentate gyrus during post-SE epileptogenesis rather than PTZ kindling. Both types of epileptogenic insults significantly promoted the proliferation of neural progenitor cells in the dentate gyrus; however, compared to 80% neuronal differentiation ratio in the control group, there was a remarkable decrease in PTZ kindling and pilocarpine models, that is 58% and 29%, respectively. Double/triple immunofluorescence labeling revealed no newborn neurons colabeled with GFP in both intact and epileptic dentate gyrus. In addition, valproate (a first-line antiepileptic drug) treatment prevented the loss of GABAergic interneurons but still failed to induce the regeneration of GAD67-positive interneurons in the dentate gyrus during post-SE epileptogenesis. **Conclusions:** These results indicate that degeneration of GABAergic interneurons may depend on the type of epileptogenic insult and that no newborn GABAergic interneurons occur in the adult dentate gyrus during epileptogenesis.

## Introduction

Epilepsy is one of the most common chronic neurological disorders with prevalence of 0.4% to 1.0% [1]. It is characterized by recurrent, usually unprovoked, epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition [2]. The current treatment of epilepsy is symptomatic by merely preventing or suppressing seizures instead of clearly influencing the process developing epilepsy (epileptogenesis).

Epileptogenesis refers to a process in which an initial brain-damaging insult (e.g., traumatic brain injury, stroke, infection, and prolonged febrile seizures) triggers a cascade of molecular and cellular changes including neurodegeneration, neurogenesis, gliosis, angiogenesis, axonal sprouting, axonal injury, dendritic remodeling, invasion of inflammatory cells, alterations in extracellular matrix, acquired channelopathies, and reorganization of the molecular architecture of individual neuronal cells [3]. A severe consequence of these changes is the imbalance between excitation and inhibition in the hippocampal neuronal network, which may cause the occurrence of spontaneous recurrent seizures (SRS). As the major inhibitory neurotransmitter in the central nervous system (CNS), GABA plays a crucial role in regulating neuronal excitability. However, the role that GABAergic interneurons may play in epileptogenesis continues to be a topic of considerable debate. On the one hand, GABAergic interneurons might be selectively vulnerable to various injuries including convulsive agents-induced epilepsy [4,5] and excessive electrical stimulation [6]; on the other hand, GABAergic interneurons are selectively spared following some other insults [7,8].

In addition, it is widely accepted that continuous neurogenesis exists in the adult hippocampus through the life of a mammal,

including humans [9,10]. The newborn neurons mostly differentiate into excitatory granular cells and functionally integrate into preexisting hippocampal neural circuitry [11–13]. However, little is known how many, if any, newborn cells in the hippocampus can differentiate into inhibitory GABAergic interneurons in the epileptic hippocampus.

For investigating the alteration of GABAergic interneurons in the adult brain, previous studies used immunohistochemistry with an antibody against glutamic acid decarboxylase (GAD) or GABA to identify the GABAergic interneurons [14,15]. However, to achieve a more precise understanding of the degeneration and regeneration of GABAergic interneurons in the epileptic hippocampus, here, we used glutamic acid decarboxylase-green fluorescence protein (GAD67-GFP) knock-in mice, in which the neurons containing GAD67, that is GABAergic interneurons, express GFP [16]. We employed two types of epileptogenesis models: One is the pentylenetetrazol (PTZ)-induced chronic kindling model [17] and the other is the lithium-pilocarpine-induced status epilepticus (SE) model [18,19]. First, we examined the degeneration of GABAergic interneurons in the dentate gyrus in two distinct epileptogenesis models. Then, we explored how many, if any, newborn cells in the dentate gyrus can differentiate into inhibitory GABAergic interneurons in the epileptic hippocampus. Last, we investigated the effect of sodium valproate (VPA), the most widely used first-line broad-spectrum antiepileptic drug, on the degeneration and regeneration of GABAergic interneurons in the epileptic hippocampus.

## Materials and Methods

### Animals

Male $GAD_{67}$-GFP knock-in mice weighing 20 to 28 g at the time of first treatment were used in this study. The generation and characteristics of the GAD67-GFP knock-in mice have been described previously [16]. The mice were housed under controlled temperature and light conditions (12-h light/dark cycle), with *ad libitum* access to food and water. All experimental procedures used were in strict accordance with the guidelines established by the U.S. NIH and were approved by the Fourth Military Medical University Animal Care Committee. All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

### PTZ Kindling Model

Mice received intraperitoneal (i.p.) injections of PTZ (Sigma-Aldrich, St. Louis, MO, USA) at a subconvulsive dose of 40 mg/kg every other day consecutively up to 4 weeks. After each PTZ injection, the mice were placed singly and were closely observed for 30 min. The intensity of convulsions was rated according to Racine's criterion (1972). When the animal had stage 4 seizures, that is clonic convulsions in the forelimbs with rearing, on three consecutive administrations, it was defined as being fully kindled and the treatment was discontinued [20]. These clonic seizures generally last <5 min. The animals usually reached the criterion of kindled seizures after 7–12 injections of PTZ. Control mice were given an equal volume of vehicle (0.9% saline) and were kept isolated as PTZ-kindled mice for 30 min. After the first PTZ or vehicle injection, all mice received i.p. injections of 5-bromodeoxyuridine (BrdU, Boehringer Mannheim, Indianapolis, IN, USA) at dose of 100 mg/kg daily for 3 days. Experimental mice were allowed to survive for 1 day and 14 days after being kindled.

### Lithium-pilocarpine Model

SE mice were generated by lithium-pilocarpine injection, as described previously [19]. An aqueous solution of lithium chloride (10 mEq/kg, i.p., Sigma-Aldrich) was injected 18–20 h prior to the administration of pilocarpine. Mice were pretreated with methylscopolamine bromide (1 mg/kg, Sigma-Aldrich) 30 min prior to pilocarpine, and then, single dose of pilocarpine (100 mg/kg, i.p., Sigma-Aldrich) was administered. Seizures were scored by Racine's scale (1972). When mice experienced stage 4 or 5 seizures for 1 h, sodium pentobarbital (40 mg/kg, i.p.) was used to terminate seizures. All mice surviving SE were video-monitored with video cameras for 8 h per day from day 10 to day 20 after SE. Only those mice that displayed spontaneous convulsive seizure activities of stage 4 or 5 were defined as chronic epileptic mice and were used for further analysis. All mice received 3 i.p. injections of BrdU (100 mg/kg per injection) once a day, beginning at day 6 after pilocarpine or saline treatment. Mice were killed 3 weeks or 8 weeks after pilocarpine administration.

### VPA Treatment

In the lithium-pilocarpine-induced SE model, a subset of mice that displayed continuous and convulsive seizure activities of stage 4 or 5 for 1 h after pilocarpine injection were used for VPA experiment. After SE termination, mice were randomly divided into two groups, so that the severity of SE did not significantly differ between groups. One group was treated with VPA (Sigma-Aldrich) and the other group received vehicle. A third group, the naive control, received vehicle injections as well. VPA was dissolved in 0.9% saline and administered as a bolus injection of 400 mg/kg i.p. five hours following pilocarpine injection. From the next day on, VPA was administered twice daily (8 a.m. and 8 p.m.) at a dose of 200 mg/kg i.p. over a period of 8 weeks. The dosage was chosen based on the previous study [21]. All mice received 3 i.p. injections of BrdU (100 mg/kg per injection) once a day, beginning at day 6 after pilocarpine or saline treatment. Mice were killed 8 weeks after pilocarpine administration.

### Tissue Fixation and Immunohistochemistry

Under anesthesia with sodium pentobarbital (50 mg/kg, i.p.), mice were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were postfixed in the same fixative for 2 h and placed in 20% sucrose until they sank. Coronal sections (30 μm thickness) through the entire hippocampus were cut on a sliding microtome, stored in PBS, and stained with different techniques for different purposes.

Immunohistochemical detection of single BrdU labeling was conducted with previously described protocols [22]. Briefly, free floating sections were pretreated in 50% formamide/2 × SSC buffer (0.3 M NaCl, 0.03 M sodium citrate) at 65°C for 2 h and were

incubated in 2 M HCl at 37°C for 30 min. After a 10-min wash in 0.1 M borate buffer (pH 8.5) to neutralize the HCl, sections were incubated in mouse anti-BrdU antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 36 h. Sections were washed again and incubated in biotinylated goat anti-mouse secondary antibody (1:250; Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature (RT), followed by wash in PBS and incubation in avidin-biotin-peroxidase complex for 2 h. After a final wash, sections were colorized by the peroxidase substrate 3, 3′-diaminobenzidine.

For immunohistochemical detection of single labeling of GFP, sections were incubated in rabbit anti-GFP antibody (1:500; Molecular Probes, Life technologies, Burlington, ON, Canada) for 18 h at RT. Sections were washed and incubated in biotinylated donkey anti-rabbit IgG (1:200; Vector Laboratories) for 2 h at RT, followed by incubation in avidin-biotin-peroxidase complex for 2 h. The specificity of immunolabeling was verified by controls in which the primary antibody was omitted.

For double or triple immunofluorescence staining, DNA in the sections was first denatured to expose the antigen as described above. Sections were then incubated at 4°C for 36 h in a cocktail solution containing the following antibodies: rat anti-BrdU antibody (1:200; Santa Cruz Biotechnology), rabbit anti-GFP antibody (1:250; Molecular Probes, Life technologies) and/or mouse antineuronal nuclear antigen (NeuN) antibody (1:500; Chemicon, Temecula, CA, USA), or rabbit antiglial fibrillary acidic protein (GFAP) antibody (1:500; Santa Cruz Biotechnology).

After multiple washes in PBS, sections were incubated for 4 h in Alexa Fluor 488-conjugated donkey anti-rabbit IgG, Alexa Fluor 594-conjugated goat anti-rat IgG (1:400; Molecular Probes) and/or Cy5-conjugated donkey anti-mouse IgG (1:400; Vector Laboratories), or Cy5-conjugated donkey anti-rabbit IgG (1:200; Vector Laboratories). Sections were mounted on gelatinized slides and coverslipped.

## Cell Counts

Every sixth section throughout the hippocampus was processed for BrdU immunohistochemistry. All BrdU-labeled cells in the dentate gyrus were counted in each section by an experimenter blinded to the study code. To distinguish single cells within clusters, all counts were performed at 400 × under a light microscope (Olympus BX-51, Hamberg, Germany), omitting cells in the outermost focal plane. The total number of BrdU-labeled cells per section was determined and multiplied by 6 to obtain the total number of cells per dentate gyrus [23]. GFP-positive cells were counted with the same method as BrdU counting.

For quantification of BrdU/NeuN and BrdU/GFP double-labeling cells, immunofluorescence images were obtained under a confocal laser scanning microscope (Olympus X2100). Fifty BrdU-

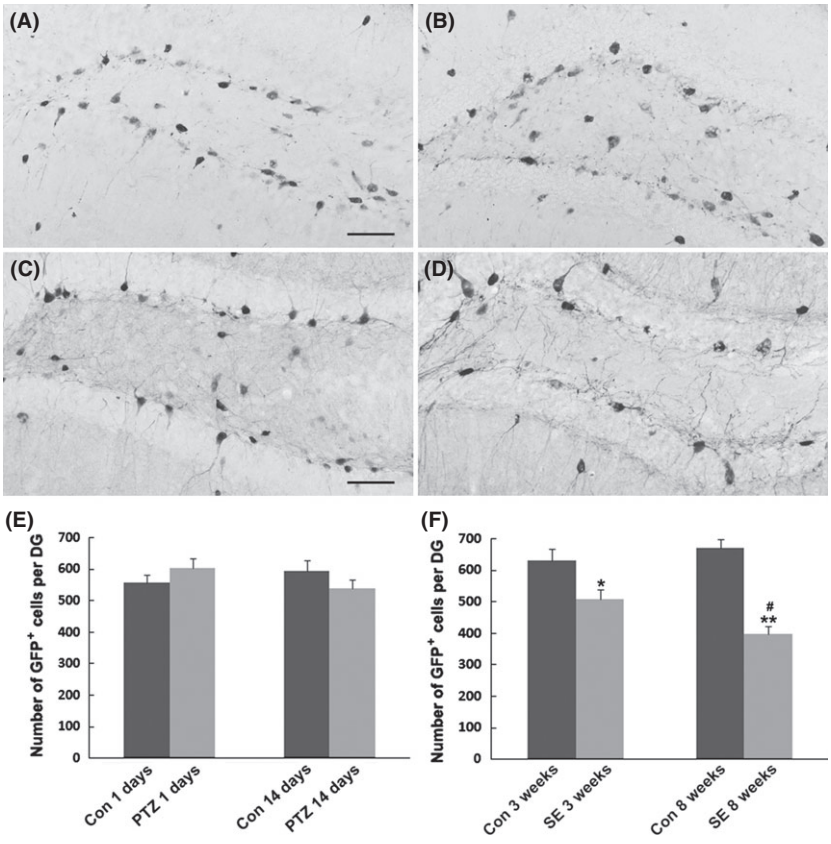


**Figure 1** Expressions of GAD67-positive interneurons in the dentate gyrus (DG) postepileptic seizures. (**A**–**B**) Representative GFP staining in the DG in the control (**A**) and PTZ kindling 14 day (**B**) mice. (**C**–**D**) Representative GFP staining in the DG in the control (**C**), and SE 8 week (**D**) mice. (**E**) No significant change of the number GFP-positive cells appeared in the DG post-PTZ kindling. (**F**) The number of GFP-positive cells in the DG was reduced in both SE 3 week and SE 8 week groups. $*P < 0.05$, $**P < 0.01$ compared to the corresponding control group. $\#P < 0.05$ compared to SE 3 week group. n = 5 mice per group. Error bars represent SEM. Scale bar = 100 μm.

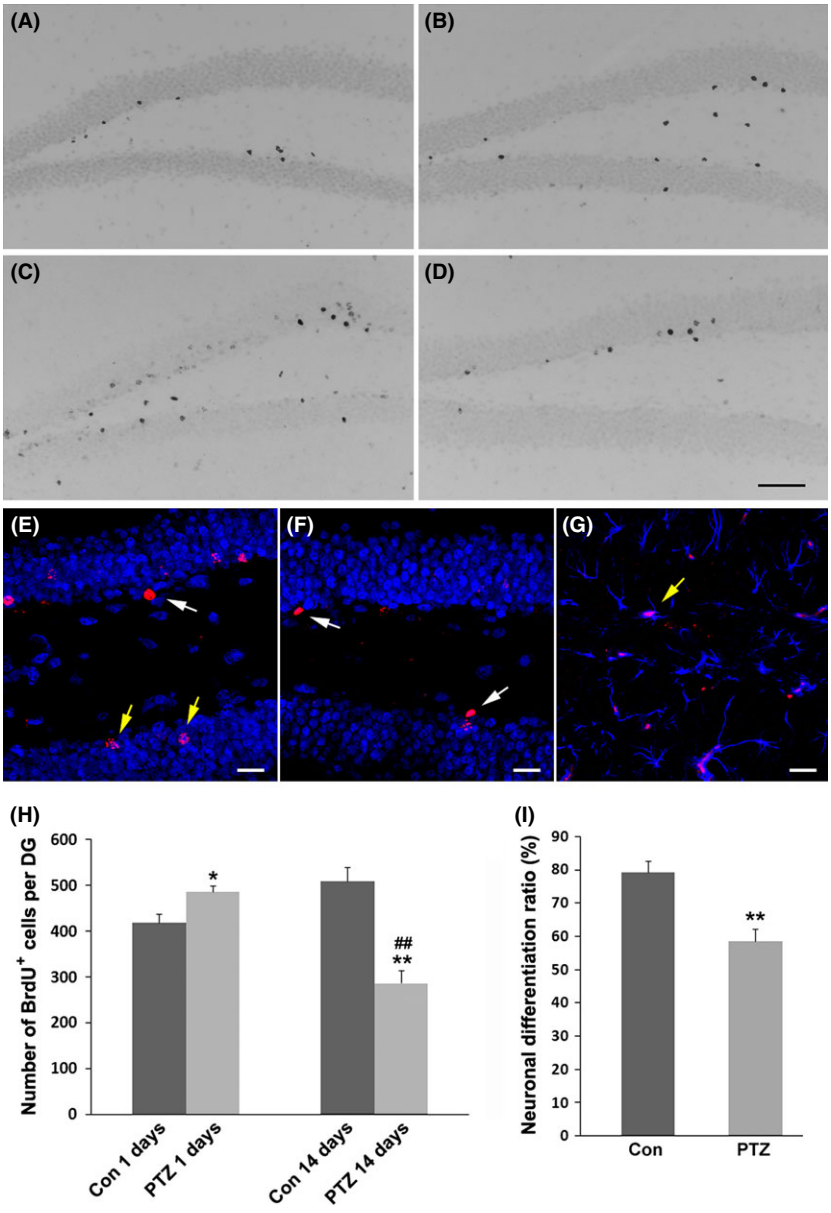


**Figure 2** Effects of PTZ kindling on the hippocampal neurogenesis. (**A**–**D**) Representative BrdU staining in the dentate gyrus (DG) of mice killed 1 day (**A**, control; **B**, PTZ treatment) and 14 days (**C**, control; **D**, PTZ treatment) after kindled. (**E**–**G**) Fluorescence images of BrdU-(red), NeuN-(blue), GFAP-(blue), and double-labeled cells in the DG of mice killed 14 days after kindled (**E**, control; (**F**–**G**) PTZ treatment). White arrows point to BrdU single-labeled cells and yellow arrows point to double-labeled cells. (**H**) Number of BrdU-labeled cells in the DG at 1 day and 14 days after kindled. (**I**) Differentiation ratio of newborn neurons in the DG 14 days after kindled. *$P < 0.05$, **$P < 0.01$ compared to the corresponding control group. ##$P < 0.01$ compared to PTZ 1 day group. n = 5 mice per group. Error bars represent SEM. BrdU was given after the first PTZ treatment. Scale bar = 100 μm (**A**–**D**); 20 μm (**E**–**G**).

positive cells in each mouse were randomly selected and then analyzed by orthogonal reconstructions from z-series (z-step, 1 μm) to obtain the proportion of double-labeling cells to BrdU-stained cells.

### Statistics

All results are expressed as mean ± SEM. One-way ANOVA followed by a Dunnett's test was used for comparing multiple-treatment groups with a control group. The two-sample Student's *t*-test was used for comparison when only two groups were analyzed. Data management and statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $P < 0.05$.

## Results

### Degeneration of GABAergic Interneurons in the Dentate Gyrus during Epileptogenesis

We first examined whether two distinct epileptogenic insults produce different effects on the degeneration of GABAergic interneurons in the dentate gyrus. In accord with the previous study [24], the majority of GFP-positive cells, that is GAD67-positive GABAergic interneurons, were located in the hilus in GAD67-GFP knock-in mice. As depicted in Figure 1, PTZ kindling did not lead to a significant change in the number of GFP-positive cells in the dentate gyrus whether on day 1 or on day 14 after kindled (Figure 1A–B,E), indicating that GABAergic inter-

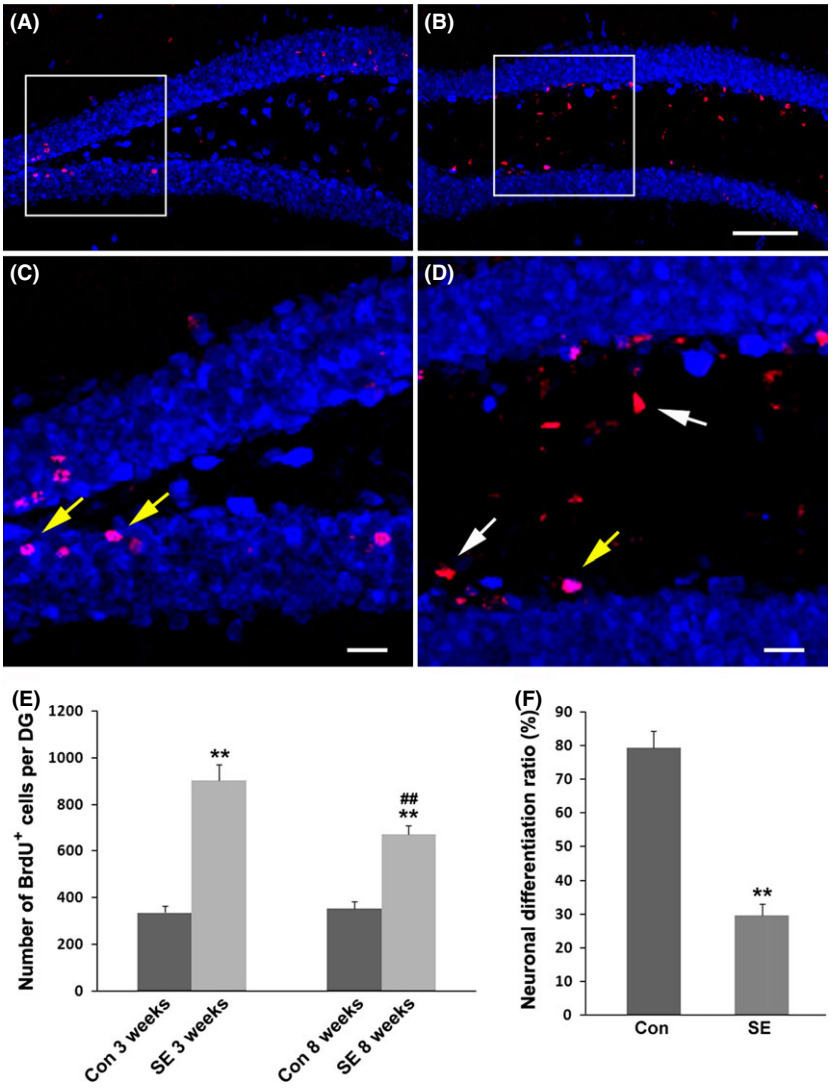


**Figure 3** Effects of pilocarpine-induced SE on the hippocampal neurogenesis. (**A**–**D**) Fluorescence images of BrdU-(red), NeuN-(blue), and double-labeled cells in the dentate gyrus (DG) of mice killed 8 weeks after SE. (**A**) control; (**B**) SE group. (**C**) High-power view of the boxed region in (**A**); (**D**) High-power view of the boxed region in (**B**). Note that most BrdU-labeled cells in the epileptic mice abnormally migrated into the dentate hilus. White arrows point to BrdU single-labeled cells and yellow arrows point to double-labeled cells. (**E**) Number of BrdU-labeled cells in the DG at 3 weeks and 8 weeks post-SE. (**F**) Differentiation ratio of newborn neurons in the DG 8 weeks after SE. $^{**}P < 0.01$ compared to the corresponding control. $^{\#\#}P < 0.01$ compared to SE 3 week group. n = 5 mice per group. Error bars represent SEM. BrdU was given 6 days after SE. Scale bar = 100 μm (**A**–**B**); 20 μm (**C**–**D**).

neurons are preserved in the PTZ-induced epileptogenesis. In contrast, pilocarpine-induced SE resulted in a significant loss of GFP-positive interneurons in the dentate gyrus in GAD67-GFP knock-in mice at both 3 weeks and 8 weeks after SE (Figure 1C–D,F). Moreover, the number of GFP-positive interneurons in the dentate gyrus was significantly lower at 8 weeks after SE as compared to 3 weeks, suggesting a progressive loss of GFP-positive interneurons in the epileptogenesis post-SE.

## Regeneration of GABAergic Interneurons in the Dentate Gyrus during Epileptogenesis

Second, we observed the effects of two distinct epileptogenic insults on the GABAergic neurogenesis in the hippocampus of GAD67-GFP knock-in mice. In the PTZ kindling model, the majority of BrdU-labeled cells were distributed as isolated cells in the dentate granular cell layer and the subgranular zone, minority of which scattered in the dentate hilus (Figure 2A–D). Quantitative analysis showed a significant increase of the number of BrdU-labeled cells in the dentate gyrus on day 1 after kindled as compared with control ($P < 0.05$, n = 5 mice per group). On 14 days after kindled, the number of BrdU-labeled cells in the dentate gyrus was significantly reduced compared to either the corresponding control or day 1 after kindled group ($P < 0.01$, n = 5 mice per group) (Figure. 2H). Since this time point (14 days after kindled) is around 4–5 weeks after the completion of BrdU injections, we continued to use BrdU/NeuN double labeling combined with confocal laser scanning microscope to determine the neuronal differentiation ratio of newborn cells in the dentate gyrus. More than 80% of newborn cells differentiated into neurons in the control group, whereas only approximate 58% of newborn cells differentiated into neurons in PTZ-kindled mice (Figure 2E–F,I). Some BrdU-labeled cells in the kindled mice were colabeled with an astrocyte marker, GFAP (Figure 2G), indicating that these newborn cells differentiated into astrocytes.

At 3 weeks after pilocarpine-induced SE, the number of BrdU-labeled newborn cells rose up to 2.7-fold of that in the intact dentate gyrus ($P < 0.01$, n = 5 mice per group) (Figure 3E).

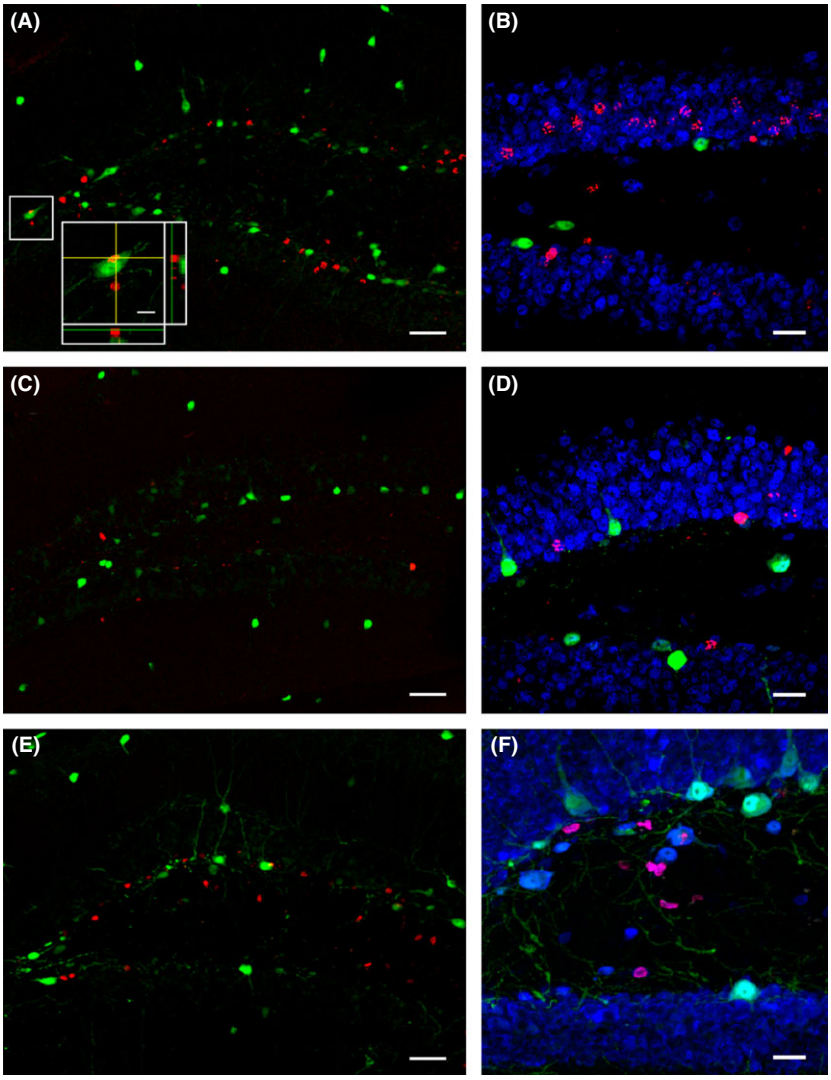


**Figure 4** No newborn GAD67-positive neurons in the dentate gyrus (DG). Representative double-labeled images of BrdU (red) and GFP (green) in the DG in the control (**A**), PTZ 14 days (**C**), and SE 8 week (**E**) mice. Representative triple-labeled images of BrdU (red), GFP (green), and NeuN (blue) in the DG in the control (**B**), PTZ 14 days (**D**), and SE 8 week (**F**) mice. Note that no BrdU-positive cell colabeled with GFP. The inset in the first panel is of a confocal 3D reconstruction of the cell in the boxed region, showing two neighboring cells rather than a double-labeled cell. Scale bar = 50 μm (**A**, **C**, **E**); 20 μm (**B**, **D**, **F**).

Then, the number declined to 1.9-fold of control at 8 weeks after SE ($P < 0.01$) (Figure 3E). In the intact dentate gyrus, most BrdU-labeled cells were located in the granular cell layer and the subgranular zone. In contrast, the majority of BrdU-labeled cells were present in the dentate hilus after SE (Figure 3A–D). In addition, the differentiation ratio of newborn neurons decreased significantly, that is only 29% of newborn cells differentiating into neurons in the dentate gyrus 8 weeks after SE (Figure 3F).

To determine the GABAergic identity of newborn cells in the dentate gyrus, we employed BrdU/GFP double-labeling and BrdU/GFP/NeuN triple-labeling immunofluorescence. As illustrated in Figure 4, we examined randomly fifty BrdU-positive cells in each mouse and did not found any BrdU-positive cells colabeled with GFP in the epileptic dentate gyrus as well as in the intact dentate gyrus. The results indicated that there were not newborn GAD67-positive interneurons in the dentate gyrus during epileptogenesis whether PTZ kindling-induced or SE-induced.

## Effects of VPA Treatment on GABAergic Interneurons in the Dentate Gyrus during Epileptogenesis

Finally, we sought to examine whether VPA treatment can protect the loss of GABAergic interneurons or induce the production of GABAergic interneurons in the dentate gyrus in epileptogenesis. Given that PTZ kindling-induced epileptogenesis failed to result in the loss of GABAergic interneurons in the dentate gyrus, we did not continue to use VPA treatment in this model. We focused primarily on the effect of VPA treatment on GABAergic interneurons in the epileptogenesis post-SE. As shown in Figure 5, pilocarpine-induced SE caused an obvious decrease of the number of GAD67-positive interneurons in the dentate gyrus at 8 weeks after pilocarpine injection. However, the epileptic mice receiving VPA treatment showed a significant increase in the number of GFP-positive cells in the dentate gyrus compared with those without VPA and displayed a level similar to those in the normal control

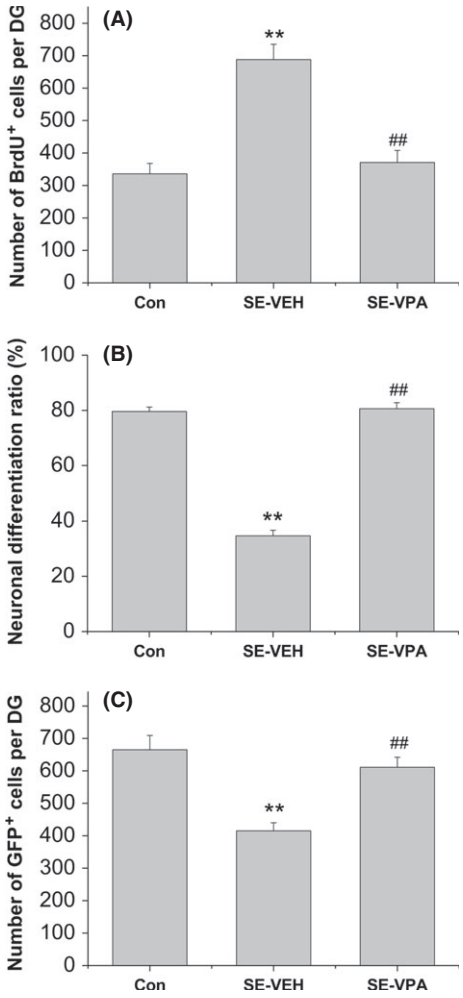


**Figure 5** Effects of valproate treatment on the hippocampal neurogenesis after pilocarpine-induced seizures. (**A**) VPA treatment inhibited the increased proliferation of neural progenitor cells in the epileptic dentate gyrus. (**B**) VPA treatment reversed the decreased differentiation ratio of newborn neurons in the epileptic dentate gyrus. (**C**) VPA treatment protected the GFP-positive GABAergic neurons in the dentate gyrus from seizure-induced injury. $^{**}P < 0.01$ compared to the control group. $^{\#\#}P < 0.01$ compared to the SE-VEH group. n = 5 mice per group. Error bars represent SEM.

(Figure 5C). This suggested that VPA treatment may effectively attenuate the GABAergic interneuron damage in epileptic dentate gyrus.

Consistent with the results in the Figure 3, pilocarpine-induced SE led to a strong increase of the number of BrdU-positive cells and a severe decrease of neuronal differentiation ratio in the dentate gyrus as compared with the normal control ($P < 0.01$). By daily VPA injections after SE, both the phenomena were reversed to a similar level in the intact dentate gyrus (Figure 5 A,B). Furthermore, we analyzed randomly 50 BrdU-positive cells in the dentate gyrus in each mouse with VPA treatment and still found no BrdU/GFP double-labeled cell, which indicated that even the first-line AED, VPA, could not induce hippocampal GABAergic interneurons production.

## Discussion

This study shows that two distinct epileptogenic insults produce different effects on the degeneration of GABAergic interneurons in the dentate gyrus. Pilocarpine-induced SE but not PTZ kindling resulted in the progressive loss of GABAergic interneurons in the dentate gyrus. While both types of epileptogenic insults affected the neurogenesis in the dentate gyrus to various degrees, no newborn cells differentiated into GABAergic interneurons. Antiepileptic drug VPA treatment prevented the damage of GABAergic interneurons in the dentate gyrus in the epileptogenesis induced by SE, but it still failed to induce the regeneration of GAD67-positive interneurons in the dentate gyrus. These data indicate the existence of different mechanisms in the development of two forms of epileptogenesis. Noteworthy, we found the lack of GABAergic neurogenesis in the adult dentate gyrus during epileptogenesis.

Epilepsy is a heterogeneous disorder. When addressing the mechanisms of epilepsy, it must be kept in mind that epilepsy has a number of subtypes, which are generally classified into focal/partial and generalized [2]. In this study, we used PTZ-induced chronic kindling model and pilocarpine-induced SE model to represent human generalized epilepsy [17,18] and temporal lobe epilepsy [19], respectively. Kindling is a form of epileptogenesis that can be induced by repetitive administration of initially subconvulsive amounts of excitatory drugs such as PTZ, which acts at the GABAA receptor and reduces GABAergic inhibition by interaction with the chloride ionophore [25,26], whereas in SE rodents, systemic administration of the muscarinic receptor agonist pilocarpine leads to a pattern of repetitive limbic seizures and SE, which is followed by a latent period that precedes the development of SRS [19]. We found that the PTZ kindling model did not alter the number of GAD67-positive neurons in the dentate gyrus, whereas pilocarpine-induced SE resulted in a progressive loss of GAD67-positive neurons. This could be explained by the fact that the two models exhibit different levels of seizure activities. The mice in the pilocarpine model experienced severe epileptic seizures (stage 4 or 5 seizures by Racine's scale) for 1 h in the acute phase and also suffered SRS in the chronic phase. In contrast, in PTZ kindling model, mice just experienced several stage 4 seizures (clonic convulsions in the forelimbs with rearing) for a few minutes. However, the progressive loss of GABAergic interneurons in the dentate gyrus could in turn contribute to development of epileptogenesis in the pilocarpine model.

In line with the previous results [27,28], this study shows that both types of epileptic seizures promoted the production of newborn cells in the dentate gyrus at early time point, that is 1 day after kindled and 3 weeks post-SE, then, induced the decrease of BrdU-labeled cells in both 14 days after kindled and 8 weeks post-SE, possibly due to BrdU dilution or cell death. However, a previous study examining multiple cell division using BrdU combined with the cell cycle marker Ki-67 staining concluded that cell division continues for up to 4 days following BrdU, and any decreases in BrdU labeling beyond this time point reflect cell death rather than BrdU dilution [29]. Mohapel et al. also reported that there was the same number of Ki-67 positive cells in the hippocampus

 

between epilepsy and control groups 5 weeks after SE [30]. Accordingly, the observed decrease of BrdU-positive cells in those periods after seizures is contributed by increasing cell death rather than BrdU dilution, which can be further explained by the fact that chronic epilepsy produces an unfavorable microenvironment for the survival of newborn cells, such as the associated inflammation in the epileptic hippocampus and the lack of appropriate trophic support and connections [31,32]. In the chronic period of epilepsy, the ratio of neuronal differentiation of newborn cells was also decreased. The underlying mechanisms might be also related to paucity of factors (BDNF, IGF-1, FGF-2, Wnt proteins, etc.) that favor neuronal differentiation of newly born cells in the chronically epileptic hippocampus [33].

Although various types of epileptic seizures have been shown to promote the neurogenesis in the adult hippocampus [22,28,34], it is unknown whether newborn cells can differentiate into inhibitory GABAergic interneurons. Previous studies have shown that all hippocampal GABAergic interneurons are born in ganglionic eminence (GE) region in lower mammals including mice [35], migrate vertically to the marginal zone (MZ) then horizontally in the lateral-to-medial direction through the cerebral MZ, finally concentrate in the hippocampal primordium by E14–E15 [36]. The cells arrived in the hippocampus perform another step of migration, populating the strata oriens, pyramidale and radiatum of the hippocampus, or settling in the hilus of the dentate gyrus during the last prenatal days and first postnatal weeks [36–38]. Although previous *in vitro* work showed that neural stem cells prepared from rat embryonic hippocampus on E16 can differentiate into both excitatory (glutamatergic) and inhibitory (GABAergic) neurons and that BDNF specifically supports the differentiation of GABAergic interneurons [39], the similar phenomenon of generating GABAergic interneurons is not confirmed *in vivo* till now. This suggests that the hippocampus *per se* may be unable to produce GABAergic interneurons although adult neurogenesis exists.

As expected, in this study, we found no GABAergic neurogenesis in the adult normal as well as epileptic dentate gyrus, which seems to conflict with a previous report [15]. By Uusing mutant Semliki Forest Virus vectors to introduce enhanced GFP into hippocampal neurons, they found that during developmental period (6 days after the last BrdU injection) rather than maturation period (6 weeks post-BrdU injection), 14% of newborn neurons in the dentate gyrus were morphologically characterized as GABAergic basket cells. More recently, by using a line of BAC transgenic mice, Zhao and colleagues [40] found a high degree of GFP-positive cells colocalized with BrdU and the newborn neuron markers, doublecortin, and PSA–NCAM at day 7 after BrdU injection. They pointed out that the GFP expression covers the whole developmental stage of newborn neurons, which fades away as neurons mature. Actually, it has been established that immature hippocampal glutamatergic granule cells express not only GABA but also all the markers of the GABAergic phenotype, such as GAD, which shut off after the completion of development [41]. Another study by Mohapel et al. also mentioned that they did not find any newborn parvalbumin-positive cells, a subpopulation of GABAergic interneurons, in the adult dentate gyrus [30]. Thus, our data together with aforementioned studies support that adult dentate gyrus fails to produce newborn GABAergic interneurons under either normal or pathological conditions. Given that we mainly focused on the hippocampal GABAergic neurogenesis in this study, we cannot rule out the possibility that GABAergic neurogenesis occurs in the other regions, such as neocortex and striatum. In addition, we did not use other specific markers (e.g., GABA, GAD65, parvalbumin, CCK, somatostatin, calretinin) to further indentify GABAergic interneurons as the presence of GFP has been demonstrated in essentially all GABAergic interneurons in the GAD67-GFP knock-in mice [16,42], which may be a weakness in this study.

VPA is one of the most commonly used antiepileptic drugs with efficacy for the treatment of both generalized and partial epilepsies in adults and children [43]. This study shows that VPA treatment can exert powerful neuroprotective effects by preventing the GABAergic interneurons damage and reversing the abnormal neurogenesis in the dentate gyrus during post-SE epileptogenesis. A previous *in vitro* study [44] showed that VPA treatment with clinically relevant concentrations stimulated the differentiation of newly generated neurons into a GABAergic phenotype, indicating that VPA might produce an inductive effect on the genesis of GABAergic interneurons from endogenous stem cells or progenitors in the brain. Unfortunately, the result was not confirmed in either intact or epileptic hippocampus with 8-week VPA administration in our study.

Taken together, although two distinct epileptogenic insults produced different effects on the degeneration of GABAergic interneurons as well as the neurogenesis in the dentate gyrus, neither of them was able to induce the production of new GABAergic interneurons in the dentate gyrus of adult GAD67-GFP knock-in mice under the present protocol. This raises a new interesting question, that is how to make newborn neurons develop into GABAergic interneurons in the epileptic hippocampus for the treatment of epilepsy.

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## Conflicts of Interest

The authors declare no conflicts of interest.

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