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Reduced abnormal integration of adult-generated granule cells does not attenuate spontaneous recurrent seizures in mice

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ABSTRACT

Epileptic seizures lead to aberrant hippocampal neurogenesis, including increased proliferation of neural progenitors and abnormal integrations of newly generated granule cells – hilar ectopic granule cells (EGCs), mossy fiber sprouting (MFS), and hilar basal dendrites (HBDs). Previous results from ablating hippocampal neurogenesis after acute seizures have been controversial with regards to the development of spontaneous recurrent seizures (SRSs). While ablation of hippocampal newborn cells was effective, a sufficient decrease of subsequent abnormal integrations in chronically epileptic hippocampus was not well-established in these studies. Evaluations of the role of aberrant neurogenesis in epileptogenesis were therefore inconclusive. In this study, we ablated the hippocampal neurogenesis by methylazoxymethanol acetate (MAM) treatment both before and after pilocarpine induced status epilepticus (SE). We found that an overall ablation of newborn granule cells and a protracted delay after the cell ablation are required to eliminate subsequent abnormal integrations, including EGCs, MSF and HBDs. However, there were no alterations in frequency, duration and severity of chronic seizures were demonstrated following this regime. The current findings provide novel evidences that an overall decrease of abnormal integrations via cell ablation cannot exert significant effects on the development of SRSs at least in the model used in this study.

1. Introduction

Neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (DG) persists throughout life in a wide variety of mammalian species. In rodent temporal lobe epilepsy (TLE) models, initial status epilepticus (SE) insult lead to augmented DG neurogenesis and abnormal integrations of newly generated granule cells, including hilar ectopic cells (HECs), hilar basal dendrites (HBDs) and mossy fiber sprouting (MFS), which potentially mediate the formation of hyperexcitable DG circuits (Parent et al., 1997; Murphy et al., 2011; Pun et al., 2012). However, studies on whether aberrant hippocampal neurogenesis contribute to epileptogenesis have produced mixed results. Some studies using pharmacological agents (Jung et al., 2004, 2006; Sugaya et al., 2010) or genetic approaches (Cho et al., 2015; Hosford et al., 2016) concluded that inhibiting the newborn granule cells leaded to reduced spontaneous recurrent seizures (SRSs), while others indicated that ablation of newborn granule cells via pharmacological agents (Radley and Jacobs, 2003; Pekcec et al., 2008) or selective hippocampal irradiation (Pekcec et al., 2011) did not alleviate chronic seizures.

Although the ablations of hippocampal newborn cells were effective, sufficient eliminations of subsequent abnormal integrations in the chronically epileptic hippocampus were not well-established in these studies. In the papers by Cho et al. (2015), Hosford et al. (2016), and Jung et al. (2004), the numbers of HECs were reduced by cell ablation treatments, while no any change on MFS was found. Papers by Jung et al. (2006), Sugaya et al. (2010) and Pekcec et al. (2007) demonstrated a reduction in HECs after ablation of hippocampal neurogenesis, but MSF was not measured. In another study (Pekcec et al., 2011), neither the HECs nor the MFS was examined. In particular, the generation of hilar basal dendrites, another common pathological integration, was poorly assessed in these studies. After suppression of the neurogenesis by endoN, an increased number of doublecortin (DCX) immunopositive cells with HBDs was observed (Pekcec et al., 2007). Paradoxically, an decrease was also found after selective hippocampal irradiation (Pekcec et al., 2011). Whether these antineurogenic treatments have effectively reduced HBDs in adult-generated mature granule cells was not examined.

Because only adult-generated granule cells develop abnormal integrations after acute seizure insult (Walter et al., 2007; Murphy et al., 2011; Kron et al., 2010), an insufficient elimination of abnormal

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integration in these studies might be rationally attributed to suboptimal experimental schemes. More importantly, it may lead to limited evaluations on the role of aberrant neurogenesis in epileptogenesis and hence contribute to the conflicting findings mentioned above.

There are at least two factors that may be responsible for the uneven alterations of abnormal integrations. First, the granule cells born before an epileptogenic insult were neglected. The recruitment of cells born a few weeks before an epileptogenic insult into pathological circuits has been well documented (Walter et al., 2007; Jessberger, 2007; Kron et al., 2010). Treatments that only targeted a subset of granule cells born after SE (Jung et al., 2004, 2006; Pekece et al., 2007, 2011; Sugaya et al., 2010; Radley and Jacobs, 2003) might hence lead to a less significant change on the subsequent abnormal integrations. Second, suboptimal time points were chosen for behavioral and morphological observations. It takes a fairly long time for newborn granule cells to adequately develop axon sprouting (Mello et al., 1993; Kron et al., 2010). An earlier time point for behavioral or morphological observations, i.e. 4–6 weeks after SE (Cho et al., 2015; Jung et al., 2004, 2006), or a shorter interval between the blocking treatments and observations (Sugaya et al., 2010; Radley and Jacobs, 2003) will not provide an optimal assessment on the effects of ablation treatments.

This study was designed to evaluate the role of the aberrant hippocampal neurogenesis in the epileptogenesis in an improved manner. An overall reduction of abnormal integrations of adult-generated granule cells was realized via the DNA methylating agent methylazoxymethanol acetate (MAM) injection both pre- and post-SE, and the seizure activities were monitored 10–12 weeks after SE.

2. Materials and Methods

2.1. Housing and Handling of Animals

Male Swiss mice were group-housed in a temperature-controlled facility with a 12 h light/dark cycle and fed standard rodent diet and water ad libitum. All experiments were approved by Xi'an Jiaotong University Animal Care and Use Committee. In handling and care of animals, the guidelines for animal research of Xi'an Jiaotong University were strictly followed. Efforts were made throughout the study to minimize animal suffering and to use the minimum number of animals. For each experimental group, 6 animals for morphological analysis and 10–12 animals for behavioral tests were used at different time points of sacrifice (the number of animal used in each experiment was shown in Table 1).

2.2. Pilocarpine Treatment

Eight-week-old Swiss female mice were used for induction. Briefly, mice were given a single subcutaneous injection of methyl-scopolamine nitrate (1 mg/kg) (Catalog No. S2250, Sigma, USA) 30 min before the pilocarpine (Catalog No. P6503, Sigma, USA) or saline injection. Then, the mice received either a single i.p. injection of 300 mg/kg pilocarpine or saline. Once status epilepticus began (defined by continuous tonic clonic convulsive seizures), mice were housed at room temperature and returned to an incubator after seizure activity stopped. Because low seizure intensity would stimulate neurogenesis to a 'physiological plasticity' level and have few pathological consequences (for review, see Bielefeld et al., 2014), which may affect the results of the current experiment, diazepam was not used to stop seizure activities after several hours of the SE. The latency (between pilocarpine injection and the occurrence of status epilepticus), maintenance time (from beginning to the end of status epilepticus) and the number of stage 5 seizures (rearing and falling with forelimb clonus) were recorded (Racine, 1972).

Table 1
The sample size in each experiment.

Experiments	Number of animal	
	Saline treatment	MAM treatment
BrdU or DCX staining at 4 w after treatment (Fig. 1)	6	6
BrdU or DCX staining at 8 w after treatment (Fig. 1)	6	6
SE analysis (Fig. 2)	10	10
FJB staining at 3 d after SE (Fig. 2)	6	6
Timm staining at 10 w after SE (Fig. 3)		
RV-GFP injection, DCX or Prox-1 staining at 10 w after SE (Figs. 2–3)	6	6
SRSs analysis (Fig. 4)	11	12
SRSs analysis (after balanced) (Fig. 4)	5	5

2.3. MAM Treatment

The mice were injected subcutaneously with MAM (Catalog No. 136-16303, Wako pure chemical industries, Ltd, Japan) at a dose of 5 mg/kg or the same-volume of saline at intervals of 48 h. The total treatment duration was 4 weeks or 8 weeks (Fig. 14A). The general appearances and weights of the animals were monitored during the treatment with saline or MAM.

2.4. BrdU Injection

To detect the effect of MAM treatment on the number of newborn cells in the SGZ, animals received single i.p. injections of BrdU (Catalog No. B5002, Sigma, USA) at 4 weeks or 8 weeks after MAM or saline injection (Fig. 1A). Morphological observations were carried out at 2 h after the injections.

2.5. Viral Vector Production and Retrovirus Injection

A retroviral vector based on the Moloney murine leukaemia virus (Shaanxi Dong Ao biosciences. LTD, China) was used as a backbone for the construction of the recombinant retroviral vector. The expression cassettes of GFP were cloned to the vector at multiple cloning sites to generate the retroviral vectors (RV-GFP) with a CMV promoter. The retroviruses were then produced in a GP2-293 packaging cell line (Catalog No. 631458, Clontech Laboratories Inc, USA), and retrovirus stocks were purified and concentrated by centrifugation and resuspended. Viral titers were determined by infection of 293 T cells (Shaanxi Dong Ao biosciences. LTD, China) according to the standard clonogenic assay procedure.

To observe the formation of HBDs, the retrovirus was injected at 2 weeks before and 2 weeks after SE (Fig. 3A). The animals were anesthetized with 5% chloral hydrate (320 mg/kg) and fixed in a steeling stereotaxic apparatus. A small hole was drilled in the skull above the intended injection sites and a highly concentrated retroviral solution of RV-GFP ($3*10^9$ CFU/ml) was stereotaxically injected through a glass micropipette (tip diameter < 40 μm) attached to a micro injector into both left and right dentate gyrus. Two injection sites were used in every side of the DG (coordinates: 2.1 or 3.0 mm posterior to bregma, 1.3 or 2.5 mm lateral to midline, and 2.3 or 3.0 mm ventral to dura, respectively), and every site received 1.5 μl of the virus solution at a rate of 0.3 μl/min.

2.6. Tissue Preparation

For immunohistochemical or histological staining and observation of retrovirus-transduced GFP signals, the animals were anesthetized with 5% chloral hydrate and perfused transcardially with 10 ml of saline initially, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min. After perfusion, the brains were removed and kept overnight in 30% sucrose in 0.1 M phosphate buffer.