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Proliferation of NG2 cells in the epileptic hippocampus

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ABSTRACT

NG2 cells are oligodendrocyte progenitor cells, and have been shown to receive synaptic input from pyramidal neurons to generate action potentials. Whether any change of these cells occurs after status epilepticus (SE) and subsequent temporal lobe epilepsy remains unknown. In the present study, the expression of NG2 was investigated in the mouse hippocampus after pilocarpine-induced status epilepticus (PISE). We showed that reactive NG2 cells were significantly increased from 1 day to 2 months after PISE. Double immunofluorescence indicated that few NG2 cells differentiated into neurons and astrocytes after PISE, whereas the number of NG2 cells was increased significantly in the stratum lucidum of CA3 area from 1 day onwards after PISE. Our results suggest that the significantly increased reactive NG2 cells from acute to chronic stage after PISE may be involved in epileptogenesis.

1. Introduction

Cells that express the NG2 chondroitin sulfate proteoglycan represent the postnatal or adult proliferative progenitors in the brain. In the mature central nervous system (CNS), NG2 cells are stable cell population and distributed in both gray and white matters (Geha et al., 2010; Keirstead et al., 1998). These cells are distinct from astrocytes, mature oligodendrocytes, microglia, and are considered as the fourth resident glial cells population in CNS representing 5–10% of the total brain cells (Otis and Sofroniew, 2008; Ziskin et al., 2007).

NG2 cells can differentiate into oligodendrocytes and are considered as oligodendrocyte progenitor cells (Kitada and Rowitch, 2006; Komitova et al., 2009). While previous studies have suggested that NG2 cells could differentiate into neurons (Belachew et al., 2003; Rivers et al., 2008; Tsoa et al., 2014) or astrocytes (Leoni et al., 2009; Zhu et al., 2008) under certain conditions, the differentiation of NG2 cells in epilepsy remains unknown. Calbindin (CB) and calretinin (CR) immunopositive cells are different neuronal populations in the hippocampus. CB is a marker for mature granule cells in the dentate gyrus (DG) while CR is used to labeled γ-aminobutyric acid (GABA)-ergic neurons within the adult hippocampus (Tang et al., 2006). CR-immunopositive cells in the subgranular zone of DG represent newly generated neurons (Dominguez et al., 2003; Tang et al., 2006; von Bohlen Und Halbach, 2007). CD11b has been used for labeling microglial cells (Konishi et al., 2017; Smolny et al., 2014). By double labeling of NG2 with difference cell markers, the nature of reactive NG2 cells in the epilepsy model could be defined.

NG2 cells are a unique glial population with typical characteristics associated with neurons, and receive excitatory and inhibitory synaptic inputs from neurons in adult mammalian hippocampus (Bergles et al., 2000) and white matter (Kukley et al., 2007; Ziskin et al., 2007). Almost a half of adult NG2 cells are synaptically connected to pyramidal neurons (Karadottir et al., 2008). NG2 cells have ion channels and transmitter receptors for signal processing (Ge et al., 2009; Karadottir et al., 2008) and therefore, may be involved in complex brain electrophysiological activities.

The rodent pilocarpine model is based on pilocarpine-induced status epilepticus (SE), which lasts for a few hours. Animals then go through a seizure-free period, i.e., "latent period". After that, animals display spontaneously recurrent seizures (SRSs) that characterizes the chronic epileptic condition (Curia et al., 2008; Wu et al., 2015). NG2 cells were detected in a variety of human CNS diseases such as traumatic brain injury (Huang et al., 2016), multiple sclerosis (Wilson et al., 2006), glioma (Gomez-Pinedo et al., 2017), but not in epilepsy. In the present

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study, we aimed to show the progressive changes of NG2 at the acute (within 1 day), latent (1 week) and chronic (2 months) stages after pilocarpine-induced status epilepticus (PISE) and to establish the relationship between NG2 cell activation and epileptogenesis.

2. Materials and methods

2.1. Pilocarpine treatment

Male Swiss mice weighing 25–30 g were used for this study. Methylscopolamine nitrate was administered 30 min before i.p. injection of pilocarpine at 300 mg/kg (Sigma) to limit the peripheral toxic effects (Wu et al., 2015). The control group of mice was injected with saline instead of pilocarpine. In the mouse model, pilocarpine-induced behavioral changes included hypoactivity, tremor, head bobbing and myoclonic movements of the limbs progressing to recurrent myoclonic convulsions with rearing, falling and status epilepticus. EEG changes in this model have been described in our previous study (Tang et al., 2004). Only those animals with behavioral changes lasted for at least 4 h were chosen for the experimental group. The animal experiment was approved by the Institutional Animal Care & Use Committee of Tan Tock Seng Hospital-National Neuroscience Institute. Effort was made to minimize animal suffering and to use the minimal number of animals throughout the study.

2.2. Immunohistochemistry for NG2 cells

A total of 36 mice was processed for the immunocytochemical study. After deep anesthesia with chloral hydrate (0.40 g/kg), six mice were euthanized at each of the following survival intervals, i.e. 1 day, 1 week and 2 months after PISE and six mice with saline instead of pilocarpine injection from each control group were euthanized. The mice were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH: 7.4), the brain was removed and kept in 30% sucrose in 0.1 M PB overnight. Serial coronal sections at 30 μm thickness were cut through the entire antero-posterior axis of the hippocampus (Wu et al., 2015). Sections were incubated with NG2 primary antibody (mouse monoclonal, 1:200, Catalog No. 05-710, Millipore, USA). In the negative control, the primary antibodies were omitted.

2.3. Western blot

A total of 30 mice was used for western blot study, five mice were euthanized at each of the survival intervals of 1 day, 1 week and 2 months after PISE respectively. Five mice with saline instead of pilocarpine injection were treated as control group. The hippocampus in the control and experimental groups was homogenized for 1 h in cold lysis buffer containing protease and phosphatase inhibitor. Lysates were centrifuged (15,000g, for 15 min) at 4 °C and supernatant was quantified by the Bradford assay (Bio-Rad). Aliquots containing 40 μg of a total protein were boiled 5 min at 100 °C and fully mixed. The aliquots were electrophoresed by 12% SDS-polyacrylamide gels and transferred to a polyvinylidene fluoride membrane (Millipore). After blocking with 5% skimmed milk for 1 h, the membranes were incubated with the mouse anti-NG2 primary antibody (1:1000) at 4 °C overnight followed by incubation with the HRP-conjugated secondary antibody. The membranes were then developed using the enhanced chemiluminescence (ECL) and exposed to photographic film. β-actin was used as a reference protein. Results were analyzed with Image J (NIH).

2.4. Double immunofluorescence labeling

Double immunofluorescence experiment was performed for NG2 and CB, CR, glia fibrillary acidic protein (GFAP) or CD11b. Six control and six experimental mice at different time points after PISE were incubated in 4% BSA for 2 h at room temperature, and then placed overnight in a mixture of two primary antibodies for NG2 (1:500) with CB, CR (rabbit polyclonal, 1:300, Swant, Switzerland), or with GFAP (1:500), CD11b (1:200) (goat monoclonal, Chemicon, Temecula, CA, USA), respectively. Sections were then washed in TBS-TX and placed in anti-mouse IgG-Alexa594 (abcam) (for NG2) (red) for 4 h followed by incubation in anti-rabbit IgG-Alexa488 (abcam) (for CR and CB), or anti-goat (for CD11b, GFAP) (green) for 2-3 h. Sections were mounted, coverslipped. Slides were firstly viewed under a fluorescent microscope (Olympus) and then observed with a confocal laser scanning microscope (Olympus Fluoview V500, Japan).

2.5. Statistical analysis

2.5.1. Image analysis

In this study, a quantitative study for immunohistochemical staining was done by using Image-Pro Plus system (Media Cybernetics, Silver Spring, MD, USA). For each animal, three sections from temporal part of dorsal hippocampus were used for cell counting. The immunopositive NG2 cells in CA1, CA3 areas and the dentate gyrus were counted in three squares with the same size and the mean density was indicated as a number per square millimeter.

The images for NG2 immunofluoresence-labeled cells in CA3 area were taken by the confocal microscope. In each image, the number of immunopositive NG2 cells in CA3 area was counted and indicated as a number per square millimeter.

The western blot bands were scanned and quantified using Image J (NIH) to measure optical density (OD). The relative expression level of NG2 was indicated as the ratio of its optical density to that of β-actin.

2.5.2. Statistical analysis

All data were presented as mean ± standard deviation (SD) and analyzed with GraphPad Prism 5.0. Statistical analysis was carried out between the control and experimental mice at different time points by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test to determine the difference among different groups of mice (Fig. 1E, E1, E2, 2B). For double immunostaining, data were analyzed using unpaired Student's *t*-test (Fig. 3D). The value of $P < 0.05$ was considered as a statistically significant difference.

3. Results

3.1. NG2 immunostaining in the hippocampus of mice with PISE

In the control mice, only a few NG2 immunopositive cells were found in different layers of CA1 and CA3 area (Fig. 1A, A1), i.e. the strata oriens, pyramidale, and radiatum. NG2 immunopositive product was also demonstrated in the molecular layer and hilus of the dentate gyrus (Fig. 1A2). The volume of NG2 immunopositive cells was, in general, small and the processes were short in the control mice. However, the increased expression of NG2 was observed in CA1 area and the dentate gyrus (DG) at 1 day (Fig. 1B, B2 E, E2) ($P < 0.01$) and 1 week (Fig. 1B, B2, C, C2, E, E2) ($P < 0.05$) after PISE and the size of NG2 immunopositive cells was also bigger than the control. The number of reactive NG2 cells was significantly increased in the stratum lucidum of CA3 area (Fig. 1B1, C1, E1) ($P < 0.01$) at 1 day and 1 week after PISE. NG2 strongly expressed cells with enlarged cell body and long multiple processes in CA1 and CA3 areas (Fig. 1D, D1, E, E1) ($P < 0.05$) as well as DG (Fig. 1D2, E2) ($P < 0.01$) persisted at 2 months after PISE

3.2. Western blot

Western blot showed increased expression of NG2 at 1 day, 1 week and 2 months after PISE when compared to the control group (Fig. 2A, B) ($P < 0.05$).

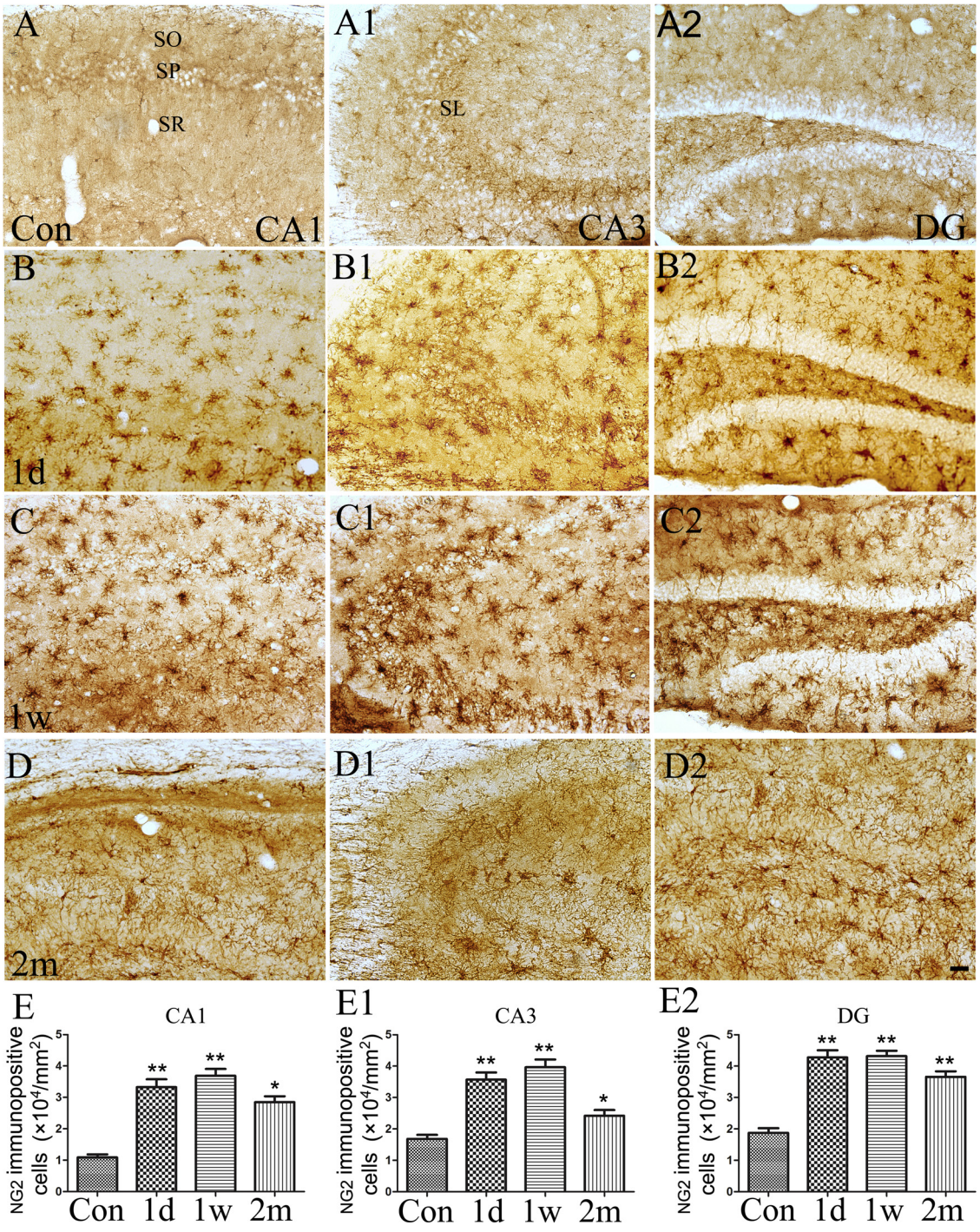

Fig. 1. NG2 immunostaining shows NG2 immunopositive cells in CA1, CA3 areas and the dentate gyrus (DG) in the control (A, A1, A2) and experimental mice at 1 day (B, B1, B2), 1 week (C, C1, C2) and 2 months (D, D1, D2) after pilocarpine-induced status epilepticus (PISE). The number of NG2 immunopositive cells in CA1 area is significantly increased at 1 day, 1 week and 2 months after PISE. Statistical analysis shows significant increase of NG2 immunopositive cells in CA1 (E), CA3 (E1) and DG (E2) at 1 day, 1 week and 2 months after PISE (*$P < 0.05$, ** $P < 0.01$). Each bar represents the average of 6 mice.
Con: control; 1 d: 1 day; 1w: 1 week; 2 m: 2 months; SO: stratum oriens; SP: stratum pyramidale; SR: stratum radiatum; SL: stratum lucidum. Scale Bar = 50 μm in D2 applies to A–D; A1–D1, A2–C2.

3.3. Double labeling of NG2 with CB, CR, GFAP and CD11b

Double immunofluorescence labeling of NG2 with markers for neurons, astrocytes, and microglia in the hippocampal sections revealed that in the control mice, there were a few NG2 immunopositive cells in the stratum lucidum of CA3 area (Fig. 3A). However, the number of NG2 immunopositive cells was increased obviously at 1 day after PISE in this region (Fig. 3B, arrowhead, D) ($P < 0.05$). In the dentate gyrus, there was no co-localization of NG2 with CB in DG at 1 day after PISE (Fig. 3C), NG2 with CR in the subgranular layer of DG at 1 day (Fig. 3E) or 2 months after PISE (Fig. 3F), and NG2 with GFAP in the stratum radiatum of CA1 area and DG (Fig. 4A, A1) at 1 week after PISE or with CD11b in DG (Fig. 3B, B1) at 1 day after PISE.

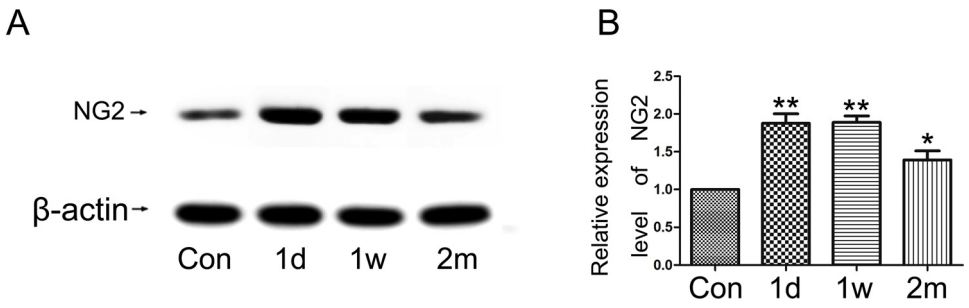

Fig. 2. Western Blot shows expression of NG2 (A, B) in the hippocampus of the control and experimental mice at different time points after PISE. β-actin is used as a loading control. Each bar represents the mean ± SD of five separate assays. *$P < 0.05$ or ** $P < 0.01$ when compared with the control.

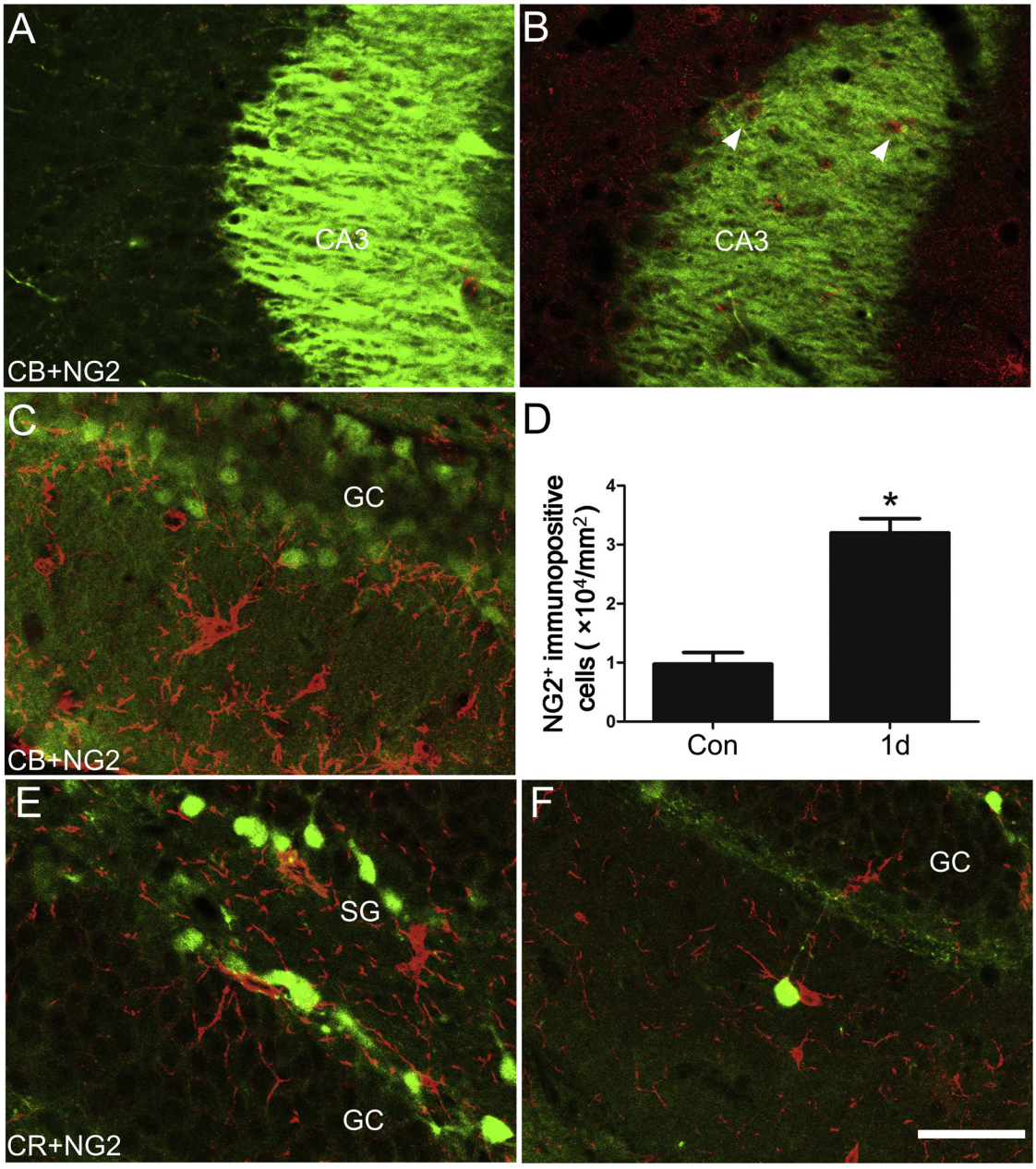

Fig. 3. Double immunofluorescence staining shows a few NG2 immunopositive cells (red) and CB immunopositive product (green) in mossy fibers of granule cells in the stratum lucidum of CA3 area in the control mice. However, much more NG2 immunopositive cells appear in the stratum lucidum of CA3 area at 1 day after PISE (A, B, arrowhead, D) (*$P < 0.05$). There is no colocalization of NG2 (red) with CB (green) in the stratum granulosum (C), with CR (green) in the subgranular layer (E) at 1 day after PISE or in the molecular layer of the dentate gyrus (F) at 2 months after PISE. GC: stratum granulosum; SG: subgranular layer. Scale bar = 50 μm in F applies to A, B, C, E.

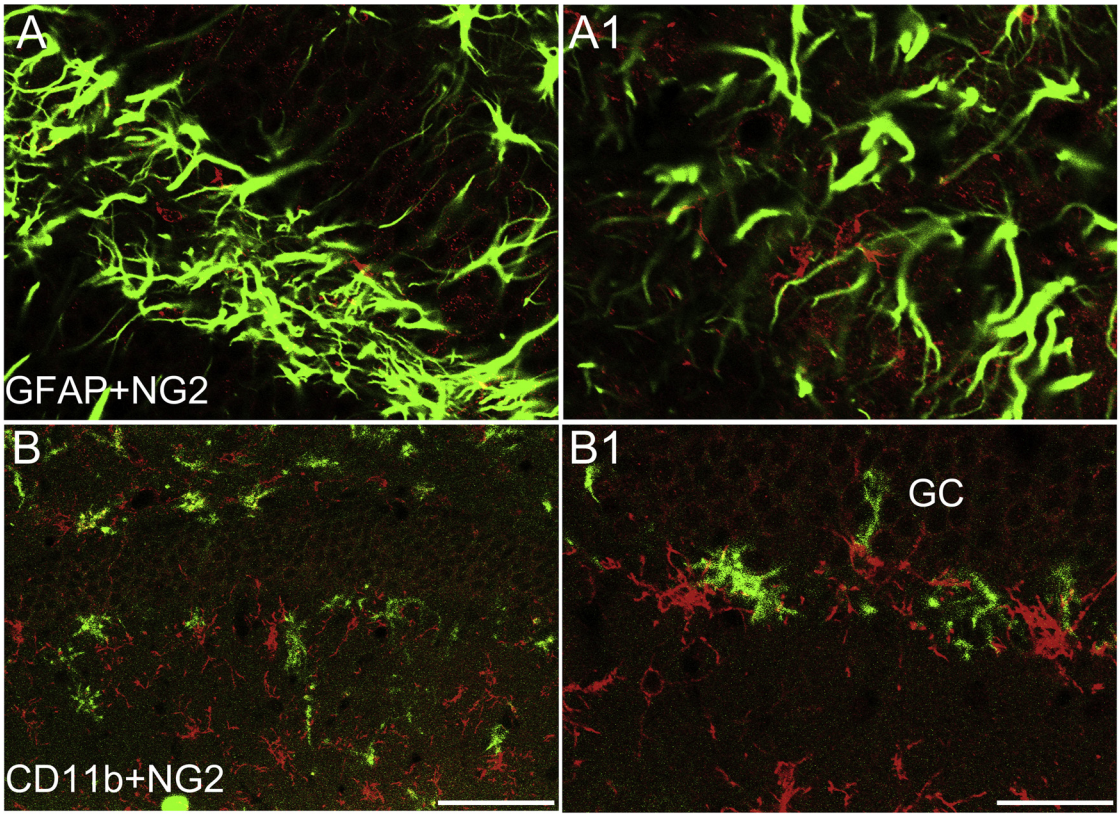

Fig. 4. Double immunofluorescence staining shows no colocalization of NG2 (red) with GFAP (green) in DG (A) and in the stratum radiatum (A1) of CA1 area at 1 week after PISE, and no colocalization of NG2 (red) with CD11b (green) in the molecular layer (B) and subgranular layer (B1) of the dentate gyrus at 1 day after PISE. GC: stratum granulosum. Scale bar = 50 μm in B applies to A; Scale bar = 70 μm in B1 applies to A1.

4. Discussion

NG2 cells play an essential role in a variety of human CNS disorders. However, it remains unknown whether there is any functional change of these cells in epileptogenesis. In the present study in the mouse pilocarpine model of temporal lobe epilepsy, a significantly increased expression of NG2 was observed from 1 day to 2 months after status epilepticus. Combined with the differentiation capability of NG2 cells and previous studies, it suggests that proliferation of NG2 cells from the acute to chronic stages after status epilepticus may be involved in the epileptogenesis.

NG2 cells may represent a specific cellular reservoir for renewal of adult inhibitory interneurons in the hippocampus (Aguirre et al., 2004). However, only few cells with co-localization of NG2 with CB or CR were observed at 1 day after PISE. It suggests that the increased expression of NG2 may not be related to the changes of CB or CR immunopositive cells. NG2 cells are also a potential source of astrocytes (Leoni et al., 2009). However, few double labeled NG2 and GFAP cells in the hippocampus of experimental mice indicate that mature astrocytes may no longer produce NG2.

Reactive NG2 cells appeared in different models of brain injuries such as viral infection, stab wound, or excitotoxin-induced epileptic seizures (Levine et al., 1998; Ong and Levine, 1999). This type of cells is the only glial cell type known to be capable of receiving synaptic connections from neuronal collaterals (Lin and Bergles, 2004). Electron microscopic analysis has demonstrated that axon terminals of pyramidal neurons make synaptic contact with NG2 cells in adult hippocampus (Bergles et al., 2000). Immunofluorescence and electron microscopic study showed that in the hippocampus, synaptic chemical transmission to NG2 cells appeared to be very similar to those made onto neurons (Lin and Bergles, 2004; Lin et al., 2005). Importantly, previous studies have confirmed that NG2 cells possess voltage-gated sodium, potassium channels (Karadottir et al., 2008) and ligand-gated ionotropic neurotransmitter receptors (Bergles et al., 2000). Electrophysiological study revealed that NG2 cells could generate action potentials (Clarke et al., 2012; Ge et al., 2009) which are similar to those produced by neurons (Karadottir et al., 2008). This finding challenges the dogma that only neurons are excitable cells in CNS and suggests that NG2 cells may be directly involved in the generation of brain hyperexcitability (Ge et al., 2006; Lin and Bergles, 2004). Combined with our study, it is speculated that SE-induced reactive NG2 cells in the hippocampus may be involved in the pathophysiological genesis of epilepsy. Mossy fibers (axons of granule cells) in the stratum lucidum of CA3 area are CB immunopositive (Tang et al., 2006). In the present study, the significant proliferation of NG2 cells in the stratum lucidum from 1 day to 2 months after PISE suggests that excitatory inputs from mossy fibers may promote proliferation of NG2 cells.

Pericytes, a pivotal cellular component of brain capillaries, are involved in vessel growth, maintenance, and pathological angiogenesis (Bergers and Song, 2005). It has been well documents that NG2 is expressed in pericytes (Klement et al., 2018; Milesi et al., 2014). In the present study, induced expression of NG2 may also occur in pericytes, leading to abnormal angiogenesis or pathological modifications of cerebrovascular system during experimental epileptogenesis (Klement et al., 2018; Marchi and Lerner-Natoli, 2013; Milesi et al., 2014).

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