Clinical and Experimental Health Sciences

Investigation of Neurogenesis in Kindled Wistar and Genetic Absence Epilepsy Rats

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ABSTRACT

Objective: The most common type of epilepsy affecting about 50 million people worldwide is temporal lobe epilepsy (TLE). Chemical and electrical kindling methods in animals can be used to form TLE model. In the present study, it was aimed to investigate neurogenesis in the hippocampus of adult kindled Wistar rats and genetic absence epilepsy rats from Strasbourg (GAERS) rats by immunofluorescence methods.

Methods: Adult Wistar and GAERS albino rats weighing 250-300 gr were injected pentylenetetrazole (PTZ) (35 mg/kg, s.c.) every other day to produce chemical kindling. Animals having 5 times grade 5 seizures were considered to be kindled. Intracardiac perfusion was performed under deep anesthesia on the 7th and 14th days after the last grade 5 seizure. Immunofluorescence methods were used to demonstrate newly formed neurons, astroglial cells, and mature neurons, by using anti-doublecortin (DCX), anti-glial fibrillary acidic protein (GFAP), and anti-neuronal nuclear antigen (NeuN) primary antibodies, respectively. Sections were then examined under a fluorescence microscope.

Results: DCX (+) cells were found to be increased in GAERS control groups compared to the Wistar control groups; and in Wistar PTZ groups compared to the Wistar control groups. DCX (+) cells were decreased in GAERS PTZ groups compared to their controls and to Wistar PTZ groups.

Conclusion: The findings of the present study suggest that the resistance to electrical kindling of GAERS reported in previous studies might be related to the increased neurogenesis in this strain.

Keywords: Doublecortin, hippocampus, kindling, neurogenesis

1. INTRODUCTION

Genetic absence epilepsy rats from Strasbourg (GAERS) is a well known animal model of absence epilepsy. In the beginnig of 1980's, researchers found out that 30% of control Wistar rats exhibited spike and wave discharges on EEG. They inbred these animals and named as GAERS (1).

Epilepsy affects 50 million people worldwide and the most common form of this disease is temporal lobe epilepsy (TLE) (2). Chemical and electrical kindling in experimental animals allow to mimic this disease (3). Pentylenetetrazole (PTZ) is one of the agents to generate TLE model.

Idiopathic generalized absence epilepsy and TLE in the same patient are rarely seen and the reason is not well understood (4, 5). GAERS strain was shown to have only stage 2 seizure, although Wistar controls had stage 5 seizure (5). The underlying mechanisms of absence epilepsy were suggested to be responsible for the resistance to secondary generalization of limbic seizures.

Neurogenesis occurs in two regions in the adult brain: Subventricular zone and the subgranular zone of hippocampal dentate gyrus. (6). Studies have shown that migrating neurons differentiate into mature, functional neurons and astrocytes (7, 8, 9). Recent studies have reported that abnormal hippocampal neurogenesis had a role in the pathophysiology of TLE (10, 11, 12).

Epileptic seizures affect neurogenesis. Acute seizures or limbic epileptogenesis stimulates neurogenesis (9, 13).

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Seizures cause some neurogenic cells to migrate (9). These neurons trigger epileptogenic hippocampal circuit (10).

Doublecortin (DCX) is a microtubule-associated protein and widely expressed during embryonic and early postnatal development in the developing neurons (14). It is widely investigated in neurogenesis studies.

In the present study, neurogenesis in the kindled Wistar and GAERS rats was investigated by using immunofluorescence methods.

2. METHODS

2.1. Animals

Adult male Wiatar albino and GAERS rats weighing 250-300 g were used in the present study. Animals were obtained from Marmara University, The Experimental Animal Implementation and Research Center. All experiments were done according to the national guidelines on animal experimentation and were approved by the Marmara University Local Ethical Committee for Experimental Animals (70.2017.mar, 09.10.2017). The animals were housed with free access to water and food in a 12-h light/dark cycle and humidity controlled room (21±2°C and 65-70% humidity).

2.2. Experimental Groups

Groups were as follows:

1. Sham-operated Wistar group 7 days (n=6)
2. Sham-operated Wistar group 14 days (n=6)
3. PTZ Wistar group 7 days (n=6)
4. PTZ Wistar group 14 days (n=6)
5. Sham-operated GAERS group 7 days (n=3)
6. Sham-operated GAERS group 14 days (n=3)
7. PTZ GAERS group 7 days (n=4)
8. PTZ GAERS group 14 days (n=3)

2.3. Chemical Kindling

Animals were injected 35 mg/kg PTZ (s.c.) every other day to induce chemical kindling. After the injection, animals were observed for 30 min and seizure development stages were recorded according to Racine's scale (15, 16). Animals having 5 times stage 5 seizures were accepted as kindled. Sham-operated groups were injected saline and observed for 30 min.

2.4. Perfusion Fixation

Animals in the PTZ group were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine hydrochloride) and perfused 7 and 14 days after the last stage 5 seizure. Then the animals were perfused with a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. After decapitation, brain tissues were obtained and incubated in the same fixative solution overnight at 4°C. Sham-operated animals were also perfused with the same method on the same days as PTZ group.

2.5. Immunofluorescence Method

After fixation, tissues were cryoprotected by incubating in sucrose solution (0.1 M phosphate buffer, pH 7.3). Then the tissues were frozen at –80ºC and 5-micron-thick sections were obtained by a cryotome. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide. Protein block was applied to prevent nonspecific staining (Skytek Superblock Solution). Sections were then incubated in anti-DCX antibody (1:4000, ab18723) to show newly formed progenitor neurons, for 1 h at 37ºC. Sections were then incubated either in anti-glial fibrillary acidic protein (anti-GFAP, 1:500, MAB3402) to show astrocytes, or in anti-neuronal nuclear antigen (anti-NeuN, 1:5000, MAB377) antibody to show mature neurons, for 1 h at 37ºC. Dylight 550 conjugated goat anti-rabbit secondary antibody (1:600, Thermo Pierce 84541) was used for anti-DCX antibody 1 h at room temperature at dark. After washing in phosphate buffer, Dylight 488 conjugated goat anti-mouse secondary antibody (1:400, Thermo Pierce 35502) was applied for anti-GFAP or anti-NeuN antibodies for 1 h at room temperature at dark. Sections were then mounted on glass slides with a mounting medium containing diamidino-2-phenylindole dihydrochloride (DAPI). Hippocampal regions were examined under a DP72 Olympus CCD camera attached BX51 photomicroscope (Japan).

3. RESULTS

3.1. Wistar Sham-Operated Groups

DCX (+) cells were seen after applying DCX-GFAP and DCX-NeuN immunofluorescence methods. DCX (+) cells were observed in subgranular zone (Figure 1a, b). NeuN (+) cells were observed in hilus and granular layer in 7 days (Figure 2a) and 14 days (Figure 2b) groups.

3.2. Wistar PTZ Groups

DCX (+) cells were observed in Wistar PTZ 7 days (Figure 1c) and 14 days (Figure 1d) groups in subgranular zone. These cells were increased both in Wistar PTZ 7 days and 14 days groups compared to the sham-operated control groups. DCX and NeuN (+) cells were observed in subgranular zone in 7 days (Figure 2c) and 14 days (Figure 2d) groups.

3.3. GAERS sham-operated groups

DCX (+) cells were observed in 7 days (Figure 1e) and 14 days (Figure 1 h) groups in subgranular zone. These cells

were observed to be increased compared to Wistar sham-operated groups. DCX and GFAP (+) cells were observed in dentate gyrus both in 7 days and 14 days groups. DCX-NeuN (+) cells were observed in subgranular zone in 7 days group (Figure 2e). Some cells in hilus showed both NeuN and DCX positivity in 14 days groups (Figure 2f).

3.4. GAERS PTZ Groups

DCX (+) cells were observed in subgranular zone in 7 days and 14 days groups and some DCX (+) cells were observed to form clusters (Figure 1g). DCX (+) cells in 14 days group (Figure 1h) were less compared to Wistar PTZ 14 days group (Figure 1d). DCX and NeuN (+) cells were observed in hilus in 7 days group (Figure 2g) and in subgranular zone in 14 days group (Figure 2h).

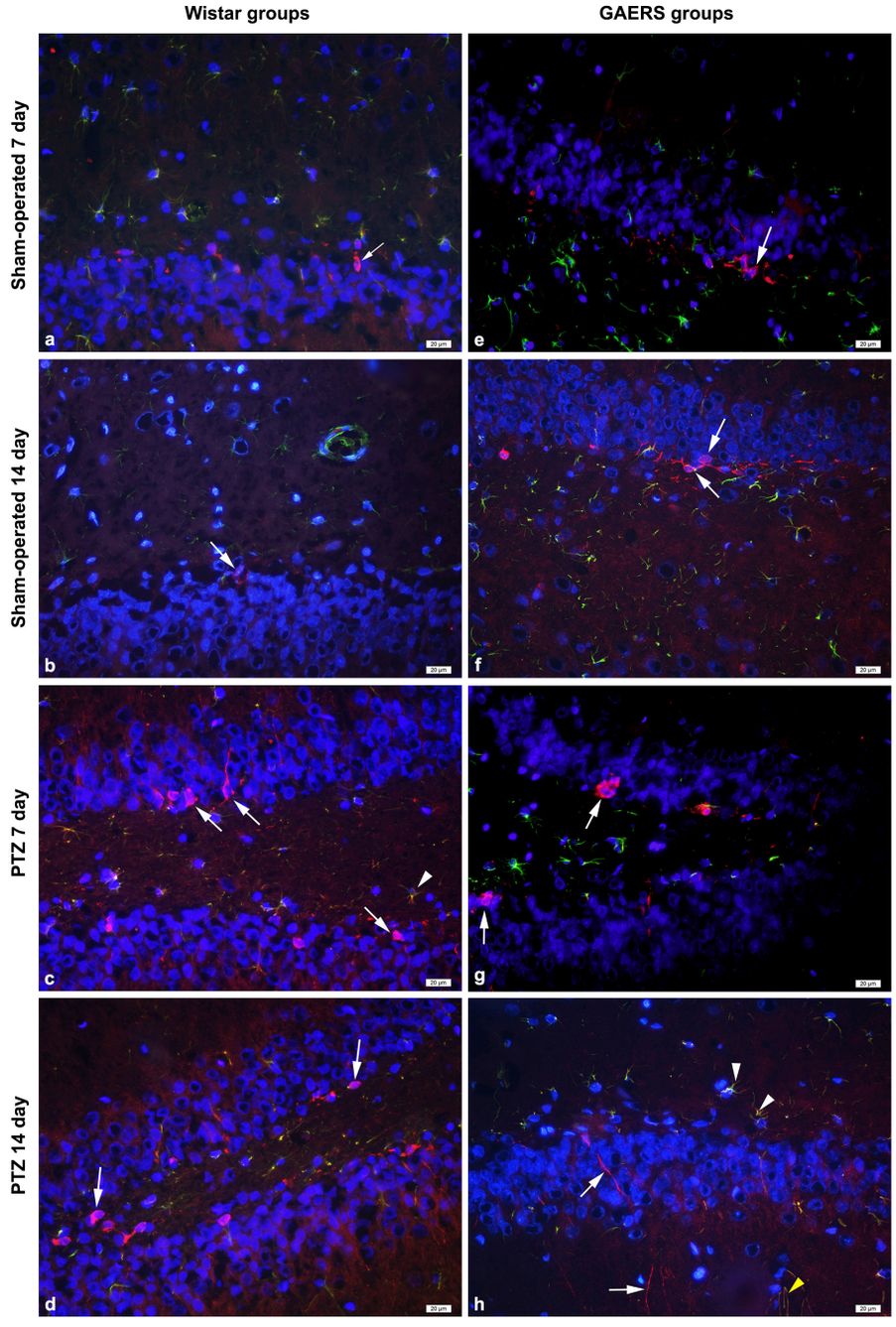

Figure 1. *DCX-GFAP-DAPI triple immunofluorescence staining. Green: GFAP, red: DCX, blue: DAPI. (a) Wistar sham-operated 7 days group. Arrow: DCX (+) cell. (b) Wistar sham-operated 14 days group. Arrow: DCX (+) cell. (c) Wistar PTZ 7 days group. Arrows: DCX (+) cells. Arrowhead: DCX-GFAP (+) cell. (d) Wistar PTZ 14 days group. Arrow: DCX (+) cells in the subgranular layer. (e) GAERS sham-operated 7 days group. Arrow: DCX (+) cell in the subgranular layer. (f) GAERS sham-operated 14 days group. Arrows: DCX (+) cells. (g) GAERS PTZ 7 days group. Arrows: DCX (+) cells. (h) GAERS PTZ 14 days group. Arrows: DCX (+) cell projections. White arrowhead: GFAP (+) cells. Yellow arrowhead: DCX-GFAP double (+) cell projections.*

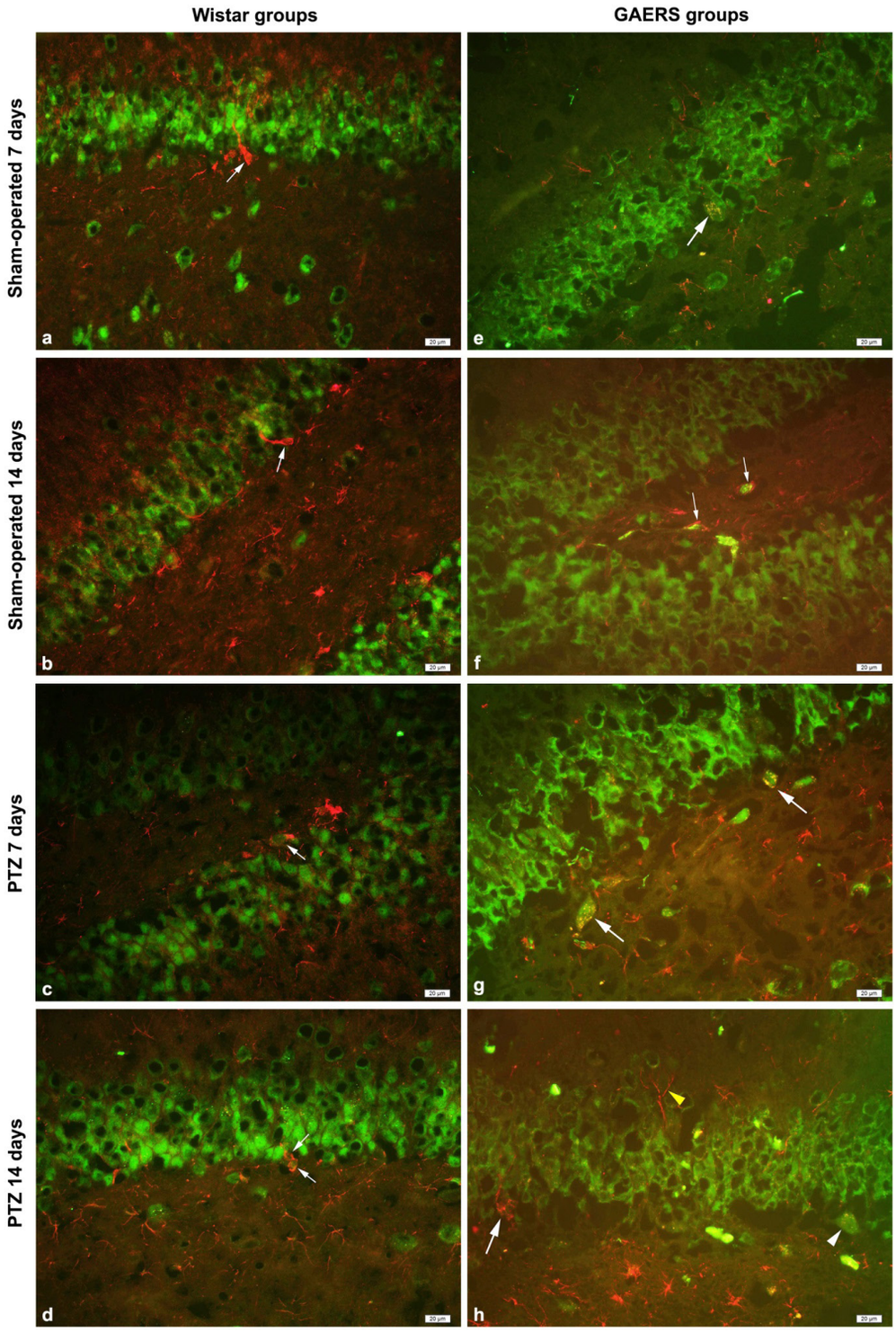

Figure 2. *DCX-NeuN double immunofluorescence staining. Green: NeuN, red: DCX. (a) Wistar sham-operated 7 days group. Arrow: DCX (+) cell. (b) Wistar sham-operated 14 days group. Arrow: DCX (+) cell. (c) Wistar PTZ 7 days group. Arrow: DCX-NeuN (+) cell. (d) Wistar PTZ 14 days group. Arrows: DCX-NeuN (+) cells. (e) GAERS sham-operated 7 days group. Arrow: DCX-NeuN (+) cell. (f) GAERS sham-operated 14 days group. Arrows: DCX-NeuN (+) cells. (g) GAERS PTZ 7 days group. Arrows: DCX-NeuN (+) cells. (h) GAERS PTZ 14 days group. Arrow: DCX (+) cell. Yellow arrowhead: DCX (+) cell projection. White arrowhead: DCX-NeuN (+) cell.*

4. DISCUSSION

In the present study, control Wistar albino and GAERS rats were injected 35 mg/kg (s.c.) PTZ and chemical kindling model was generated. Neurogenesis was found to be increased in Wistar PTZ groups compared to Wistar sham-operated groups and

in GAERS control groups compared to Wistar control groups. It was decreased in GAERS PTZ groups compared to their controls and to Wistar PTZ groups.

Neurogenesis is observed in subgranular zone of the hippocampal dentate gyrus and in subventricular zone in the adult brain (6). In our study, in parallel with the previous studies, DCX (+) cells were observed in the dentate gyrus in all groups and these were suggested to be the newly born neural progenitor cells.

GAERS strain rats were shown to resist electrical kindling previously (5). However, in the present study, we observed that GAERS rats were not resistant but more susceptible to PTZ kindling compared to Wistar rats. PTZ is a GABA-A receptor agonist and is used to generate epileptic seizures in experimental animals (17, 18). In GAERS rats, extracellular GABA was shown to be increased by microdialysis method in the thalamus (19, 20). Because there is an increased GABAergic mechanisms in this strain, GABAergic system may have been supressed and seizure susceptibility might have been increased. In a previous study, GAERS were found to be susceptible to GABA agonists, and that they needed less dose compared the controls (21). Our findings were in line with this study and GAERS rats needed less number of PTZ injections and had grade 5 seizure. Similarly, in GAERS, GABA withdrawal syndrome was generated and it was reported that convulsive seizures induced by PTZ injections were more in GAERS (22). Researchers related this cortical vulnerability induced by GABA withdrawal and PTZ to spike and wave discharges.

TLE models induced by kainic acid and pilocarpine showed cell proliferation in hippocampal dentate gyrus (8, 23). However, neurogenesis is decreased in chronic TLE (24). In animal models of absence epilepsy, including gamma-hydroxybutyrate, AY-9944 and WAG/Rij, different results were obtained (25, 26). The reason might be the strain differences. Scott et al. observed no difference between absence epilepsy group and control group in terms of neurogenesis; however, they observed increased neurogenesis in kindling group, which is a TLE model (25). In that study, it was concluded that spike and wave discharge frequency in absence epilepsy might not be sufficient for stimulating increased neurogenesis. Cell increase in kindling model in the same study was related to cell death. However, cell death was not observed in absence epilepsy. In our study, we used GAERS rats as an absence epilepsy model and we observed more DCX (+) cells compared to Wistar controls and concluded that neurogenesis was increased in this model. The reason for the different findings in the present study compared to that of Scott et al. might be that different absence epilepsy models and histological methods were used in these two different studies. Scott et al. used BrdU to show neurogenesis and TUC-4 as a marker for neurogenesis (25).

In the present study, it was observed that Wistar PTZ groups had more DCX (+) cells compared to their control groups. However, in PTZ GAERS groups, when compared to their sham-operated control groups and to PTZ Wistar groups, less DCX (+) cells were observed. The reason for this might be that GAERS reached stage 5 seizure in less time compared to Wistar rats and there may not be sufficient time for neurogenesis. Our findings suggest that seizure type may also be a factor affecting neurogenesis. Our study, showing the relation between GAERS and neurogenesis, fills a gap in the literature. To our knowledge, there is no study showing neurogenesis in GAERS.

In a previous study, it was shown that mature astrocytes also express DCX in human neocortex in some disease conditions (27). This dual presence of GFAP-DCX in astrocytes was suggested to play a role in glia-neuron communication. In the same study, it was also reported that DCX was expressed as punctate-like structures in the progenitor cells. In line with these previous studies, we observed DCX and GFAP (+) astrocyte like cells in all groups. The function of GFAP in multipotent neural progenitor cells in the subgranular zone is not known. In our study, we observed DCX and GFAP (+) cells in the Wistar PTZ 14 days group and that these cells showed bipolar morphology. Similarly, Garcia et al. reported that adult multipotent neural progenitor cells expressing GFAP were the major source of *in vivo* adult neurogenesis and they showed bipolar or unipolar morphology, and had less projections compared to non-neurogenic multipolar astrocytes (28). These findings suggest that GFAP expressing neural progenitor cells are different from astrocytes. In the present study, we observed that both neural progenitor cells and mature astrocytes showed the dual presence of DCX and GFAP.

In a previous study, DCX (+) cell clusters were demonstrated in the subgranular zone of dentate gyrus in the first days after BrdU injection (29). Similarly, we observed DCX (+) cells in clusters in GAERS PTZ group. Brown at al. reported that these cells had no distinct cell projections and the others resembled neuroblasts and their projections were parallel to the granular layer. In the same study, it was observed that DCX (+) cells were integrated into the granular layer 7 days after BrdU injection and they projected towards molecular layer. Similarly, we observed that DCX (+) cell projections projected towards molecular layer by passing through the granular layer. These dendrite-like DCX (+) projections were more distinct in the PTZ groups. This finding suggests that newly formed neurons in PTZ groups contribute to the neural plasticity.

DCX-NeuN coexistence was reported in previous studies in adult brain of the rodents (29). Brown et al. reported that NeuN (+) cells started expressing DCX 10 days after BrdU injection and this expression lasted until 14th day, and double expression was not observed after 14th day. Another study reported that 8% of newly born neurons also expressed NeuN (30). Liu et al. did not observe coexistence of DCX-NeuN in normal human brain; however, in the epileptic temporal lobe cortex, 40% of DCX (+) cells were also positive for NeuN (31). Based on these findings, they concluded that neurogenesis was increased in epileptic brain and newly formed neurons were more mature than that of in the normal brain. In our

study, we observed DCX-NeuN dual presence in the same cell both in kindling and control groups in GAERS strain. We concluded that these cells might be in a more mature stage in their development, and DCX-NeuN (+) cells in the hilus might have migrated to hilus after being formed in SGZ. Moreover, Scott et al. observed dual presence of TUC-4, a marker for progenitor neurons, and NeuN (25).

5. CONCLUSION

In conclusion, our findings showed increased neurogenesis in the sham-operated genetic absence epilepsy rats compared to sham-operated Wistar controls, and this result suggested to be related to their resistance to electrical kindling. Besides, DCX presence both in mature astrocytes and in neural progenitors, suggests that marker for this protein is not specific to neurogenesis. We suggest that when used in neurogenesis studies, marker for this protein should be accompanied by the use of other newly born neuron markers.

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