Review of: "Impending role of hippocampal neurogenesis in the development of chronic epilepsy following seizures after Kainic acid and Pentylenetetrazol treatment"

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Potential competing interests: No potential competing interests to declare.

This study challenged one of the biggest questions in epilepsy: the role of aberrant hippocampal neurogenesis in epileptogenesis. However, the methodology in this study does not seem fully constructed for its intended purpose. First, why did the authors investigate two kinds of animal models, KA-treated and PTZ-treated rats? The authors analyzed these animal models respectively and sometimes compared them. What did you expect to receive by this method? Next, the investigation of aberrant hippocampal neurogenesis is not sufficient. The data relating to neurogenesis is now only from BrdU/Calbindin double staining. Hippocampal proliferation indicated by BrdU is not enough to assess neurogenesis because glial proliferation is common in epileptic lesions. Moreover, neurogenesis is commonly investigated with the use of immature neuronal markers such as doublecortin and NeuroD. A feature of aberrant neurogenesis in epilepsy is the abnormal morphology and distribution of immature neurons.

The text should be corrected structurally overall. Avoid repetition and make it concise. In addition, there are numerous careless errors. Authors have a responsibility not only to write, but also to brush up their writing. I also recommend receiving commercial English proofreading. Further, the points to be confirmed are listed below.

Major points

1. The title seems not to be exact for the purpose of this manuscript.
   The term “impending role” seems to indicate an exacerbating effect in the development of chronic epilepsy. However, in the Discussion section, the authors conclude, “Increased neurogenesis was observed in kindling, but the development of chronic epilepsy was not observed; this suggests that aberrant neurogenesis does not contribute to the development of recurrent seizures.” Which is the author's opinion?
2. “Cell differentiation” section in Results
   BrdU/Calbindin double staining should be provided with figures, including photomicrographs and bar graphs, because this is the only data about neurogenesis, and this study aimed to investigate aberrant neurogenesis. In addition, histology should be described in more detail (distribution and cell morphology) to investigate whether the neurogenesis is aberrant or not.
3. Methods should be provided in detail, at least the important steps. Immunohistochemistry (including double staining), western blotting, Nissl and Fluoro-Jade B stains, and TUNEL have been included in this study.

4. Describe the protocol for BrdU administration. At least, the dosage, frequency, and timing of administration should be provided because this information is necessary to interpret the results.
5. Figure 1

   These figures are difficult to recognize because of the dark background. I recommend adding auxiliary lines that indicate the granule cell layer of the DG. Please also clarify what the white arrows indicate?
6. Figures 2, 3, and 4

   These graphs consist of three groups. But when the description of the control group in the Materials and Methods shows that the authors prepared a control group for KA-treated and PTZ-treated animals, respectively, these figures should include 4 groups (control for KA-treated, KA-treated, control for PTZ-treated, PTZ-treated). In addition, I recommend providing representative images of sections to ensure the reliability of the results. It should also be provided what asterisk marks mean.
7. Figure 5

   I cannot identify BrdU-positive (gray-colored) signals. Please indicate by arrows. In addition, the method for double staining should be provided. Particularly, the method for positive labeling by the gray color should be explained in detail because this is not common.
8. Figure 6

   Same with my comment #4, Fig. 6B should include control groups for KA and PTZ, respectively. It also should be provided what asterisk marks mean.
9. About data comparison
10. Occasionally, this manuscript presented the data comparison between KA-treated and PTZ-treated groups. However, it is questionable to simply compare these groups because these animal models were created in very different ways.
11. Figure 7

    For immunohistochemistry data, please provide labels for NGF and BDNF. In addition, the histological images show diffuse positive signals (all cells and neuropil in the view look brown), but the main text (Results section) describes that positive cells were pyramidal neurons in the DG.

Minor points

Generally

1. Origin of the materials used in the study should be declared. If authors bought materials, manufacture information should be provided.
2. Abbreviations should be provided in parentheses after the full name when they first appear. For example, BrdU, GFAP, and NGF are all abbreviations.
3. “After completing the drug treatment, rats from the KA and PTZ groups were divided into two sets.” How many animals were included in a group?

Introduction

None.

Materials and Methods

1. Using GFAP as a general glial marker seems to cause misunderstanding. GFAP is commonly expressed in astrocytes, but not in microglia and oligodendrocytes. Neural stem cells also express GFAP.
2. Using Calbindin as a general neuronal marker also causes misunderstanding. It is expressed in specific neurons. In the hippocampus, Calbindin is expressed mainly in granule neurons in the DG.
3. “Results were expressed as the average number (mean±S.E.M) of BrdU-positive cells per section.” Were only BrdU-positive cells expressed as mean±SEM? What about other data like the number of apoptotic cells in Figures 2, 4, 6?
4. ” Cells were counted in the CA1, CA2, CA3, hilar, and DG subfields…”
   When I see the Results section, the results of cell counts are combined in one. If the authors had cell counts in each subfield respectively, results should be presented for each subfield.

Results

1. “A large number of healthy cells with stained nuclei and nucleoli were observed in the control brain. Degenerating cells … In the PTZ-treated brain, a comparatively low number of degenerating cells was observed, and there was no change in DG morphology”
   These descriptions are about Nissl staining specimens? And, if so, I am not sure that this staining is adequate for evaluation of neuronal degeneration.
2. “A large number of healthy cells with stained nuclei and nucleoli…”
   What does “healthy” mean? Morphologically normal?
3. “In the PTZ-treated brain, a comparatively low number of degenerating cells was observed.”
   Which regions were degenerating cells present in PTZ-treated animals? What kinds of cells were degenerated?
4. “A significant increase in Fluorojade B-positive neurons was observed within the Dentate Gyrus…”
   Please use the abbreviation for the Dentate Gyrus, because this word has already been used repeatedly. Please check the entire manuscript.
5. Fluorojade B -> Fluoro-Jade B
   Please check the entire manuscript.
6. “A significant increase in Fluorojade B-positive neurons was observed within the Dentate Gyrus compared to the control group…”
   Which group did the authors mention? KA-treated or PTX-treated?
7. “…signs of mild activation of glial cells indicating a localized neuroinflammatory response…”
   How did authors identify “activation of glial cells”?
8. “The number of Fluorojade B-positive neurons appeared to decrease compared to the 48-hour time point, indicating that some neurons may have regenerated or repaired over time.”
   I think that the decreased number of Fluoro-Jade B-positive cells was a result of neuronal death and removal of dead cells. It seems to be unable to investigate neuronal regeneration by Fluoro-Jade B staining because this staining is for

detecting degenerating neurons. For neuronal repair, I wonder whether neuronal degeneration indicated by Fluoro-Jade B staining is reversible or not.

9. “…were observed within this specific hippocampal subregion.”
   Clarify the subregion. CA, DG, or other regions?
10. “After 8 weeks of SE or kindling, a significant…”
    It is better to identify the group as KA-treated or PTZ-treated. Using SE or kindling would be confusing. Please check the entire manuscript.
11. “Among the total differentiated cells counted, 73.03% were identified…”
    How were the differentiated cells identified, and how were the total differentiated cells counted?
12. “Neurogenesis was 340.20% ($P<0.001$), whereas gliosis was 235.39% ($P < 0.001$)”
    What does the percentage mean? Why do these results reach more than 100%?
13. “NADPH-d positive cell count” section
    Here figures 4 and 5 are referred to in the main text. This must be an error.
14. “The cell number was higher in the CA1 and CA3 regions compared to CA4. Few NADPH-d positive cells were also observed in the hilus and granular cell layer within the DG.”
    Which group are these sentences about? Control? In addition, the data on cell number should be provided.
15. “A statistically significant increase ($P < 0.05$) in the number of NADPH-d positive neurons was observed in both the KA (10.40±1.65) and PTZ (8.5±1.3) treated brains when compared with the control brain (5.24±0.98; Fig. 4B) after 48 hr of SE and kindling”
    Immediately before, the authors describe that the number of NADPH-d-positive cells was different (maybe in the control group). If so, it is important to clarify the regions where changes occurred in the brains of KA-treated or PTZ-treated animals.
16. “GABA immunostaining” section
    There are some significant changes, but no data is provided (no figures, no values).
17. “In control rats, BDNF and NGF immunoreactivity was present in pyramidal neurons and granule cells of DG.”
    Are pyramidal neurons distributed in the DG? Commonly, pyramidal neurons are predominant neurons in the CA regions, and granule neurons are distributed in the DG primarily. Moreover, please write “DG,” not “Dg.”
18. “BDNF expression was higher in KA- and PTZ-treated brains compared to control brains after 48 hr of KA or PTZ administration. After 8 weeks of administration, BDNF expression decreased. Similar results were obtained in the immunoblotting study.”
    How to estimate the BDNF expression in immunohistochemistry?
19. “Similar results were obtained in the immunoblotting study.”
    “Were” is duplicated.
20. “An increase in NGF immunoreactivity was observed in KA (Fig. 6)”
    What does “an increase in immunoreactivity” mean? Increased intensity of positive signals?

Discussion

1. “The present study showed that systemic administration of KA and PTZ elicited seizures in rats, but marked differences were observed in intensity, duration, and frequency.”
   There is no description of seizure induction in the results section.
2. “…by the TUNEL assay, suggesting that cell death takes place primarily by necrosis.”
   Recent studies suggest a variety of cell death mechanisms. For example, regulated necrosis (necroptosis, pyroptosis, ferroptosis) and autophagic cell death. Therefore, it should be avoided to judge necrosis because it is not apoptosis.
3. “After 8 weeks, few degenerating neurons were…”
   May “a few” be correct?
4. “The differences in the magnitude and mechanism of neurodegeneration and in seizure types after administration of KA and PTZ can be explained by their different modes of action on hippocampal neurons. KA induces significant excitotoxicity by selectively activating KA receptors in the hippocampal CA1 and CA3 subfields, which are preferentially expressed on CA3 pyramidal neurons[18].”
   What is the difference in the action of KA and PTZ? There is no explanation about the action of PTZ.
5. Fourth paragraph of Discussion
   What conclusions can be drawn from here?
6. “We hypothesize negative regulation of GABA on Neuronal Progenitor Cells (NPCs) proliferation, and a GABA antagonist like PTZ may promote NPCs proliferation”
   I cannot understand the author’s way of thinking about this hypothesis. More careful explanation is needed. In addition, this study conducted a cell proliferation assay by detecting BrdU, a molecule taken up by proliferating cells. As described in the results section, the brain lesion showed glial activation, commonly associated with glial cell proliferation. It seems to be difficult to evaluate the proliferation of NSCs or NPCs.
7. “However, PTZ lasts for only two hours in the body.”
   A reference is needed. Does the dosage not affect?
8. “A proliferative surge occurs in NSCs of the SGZ shortly after seizures,…”
   BrdU assay may be inadequate for evaluation of NSC proliferation.
9. FGF-2, VEGF
   It should be avoided to use abbreviations if the term does not appear repeatedly.
10. “These results establish a positive correlation between growth factor expression and cell proliferation.”
    Correlation should be supported by statistics. For example, Spearman’s rank correlation coefficient is famous.
11. “…, a phenomenon reported by Reitze et al., 2000”
    The referenced paper should be cited by the number in the reference list.
12. Twelfth paragraph of Discussion
    This information should be included in the Materials and Methods section, not in the Discussion.
13. “We identified nNOS-positive cells using a histochemical method for NADPH-d [24]”
    NADPH-d staining can detect NOS activity but cannot identify the kinds of NOS. Neurons have been reported to express iNOS, in addition to nNOS. In addition, the term “xxx-positive cell” should be defined as “a cell showing positive signals when you detect xxx”. When you find a cell showing a positive signal for NADPH-d staining, it is a

NADPH-d-positive cell, not nNOS-positive.

14. “Spontaneous recurrent seizures (SRS) were observed in the KA-treated brain but not in the PTZ-treated brain. This can be attributed to gliosis, which occurred only in the KA-treated brain shortly after its commencement.”
    Why do authors think so? How to exclude other factors. For example, authors described neuronal degeneration is severe in the KA-treated brain than in the PTZ-treated.
15. “In conclusion, we assert that initial seizures are not the result of neurodegeneration, as seizures were also observed in PTZ-treated rats. The onset of seizures in both KA and PTZ models may be the result of a neurotransmitter imbalance”
    This consideration would not be necessary because authors can know the pharmacological action of KA and PTZ.
16. Seventeenth paragraph of Discussion
    I do not understand what the author's intention was in this paragraph. Summary of the discussion? If so, it should be the last one.