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RESEARCH ARTICLE

Liubov Krivoshapkina et al, *TheExperiment, 2017,Vol 42(1),2418-2427*

Expression of miR-134-5p with neuronal migration protein doublecortin (DCX) in a mature epileptic animal model

Objective:

Epilepsy is a common neuropsychiatric disease with a chronic latent period, manifested in the susceptibility of the organism to the sudden appearance of convulsions. The restructuring of neuronal circuits, which produces a lesion with increased excitability, is called epileptogenesis. The formation of new neurons in the hippocampus based from dividing progenitor cells in the mature grain of new cells. The expression of neurons is formed, some of them are involved for survival in the long-term integration into the network, and this process depends on the regulated activity (Kempermann g, et al., 2003). Hippocampal neurogenesis in the adult appears to occur from astrocytes as a precursor cells (type-1 cell/entrical astrocyte) resulting in temporally amplifying progenitor cells (type-2 cell) (Sery, et al., 2001; Filippov. V., 2003; Tompson Plümpe., 2006). The majority of the GABA-ergic interneurons are instead generated outside of the cerebral cortex, in the ganglionic eminences of the basal forebrain (Wonders et al., 2006). In most cases, they are caused by violation of the neuronal migration of primary matrix (fetopathy), at later stages the violations of the neuronal migration are microstructural disorders (embryopathy). MicroRNA-134 is an important regulator of the ventral functions of neuronal precursors during neurogenesis and nervous system, detection of specific microRNAs acting as regulators of protein expression at different stages of neurogenesis. Induction of epilepsy in the hippocampus of the brain and the mechanism of expression of microRNAs are still unknown. It was noted that microRNA-134 regulates cell migration in vitro and down-regulates neuronal migration protein - Doublecortin (DCX) in vivo, thereby impairing neuronal migration (Gaughwin et al. 2011). Doublecortin (neuronal migration protein, also known as doublecortin or lissencephalin-X) is a microtubule-associated, domains serve as protein-integration platforms and regulates neuronal migration during development (Crespel A., 2005). The mutation of doublecortin gene is a result in abnormal neuronal migration such as epilepsy. The neurogenesis of the adult hippocampal regulation occurs at the level of doublecortin expression, as pro-proliferative effect on precursor cells or the survival stimulating effect on postmitotic cells. DCX protein has the leading process of migrating neurons, where subcellular localization is supported by the phosphatase and kinase activities. The microtubule binding activity of DCX is subject dynamic regulation because of phosphorylation of the N-terminal microtubule binding domain by the serine-threonine kinases (Bruce T. Schaar, et al., 2004). DCX is a marker of newly born neurons, used to determine the levels of neurogenesis in the adult dental gyrus.

Experimental animals: Animals were approved by the Institutional Animal Care and Use Committee of China, and Institutional guidelines for animal welfare. Fifty adults male Sprague Dawley white rats (230-350g) were obtained from the specific pathogen – free (SPF) Laboratory animal center of Dalian Medical University (Dalian, China). The rats were housed in a room, kept on an alternating 12 hours light-dark cycle at the Experimental animal center of Dalian Medical University. Animals had free access to water and food.

Rat model of epileptic tolerance: Animals were randomly divided into four groups each consisting of eight rats: control group and epileptic rats model, were separated into: 24 hour group, 3 week group, and 8 week group. For the experimental procedures, was used pilocarpine model. Animals were injected intra-peritoneally with lithium chloride based on the 125 mg/kg, dosage in order to reduce peripheral cholinergetic effects. After 17 hours after injection with lithium chloride, the rats were intraperitoneally-administered pilocarpine 25mg/kg. The severity of convulsion was evaluated by Racine's scale and rats with the score of 4-5 were considered. All the animals reached 4-5 rating in Racine's scale. These rats received chloral hydrate 10% - 3ml/kg intraperitoneally to terminate epileptic attacks. During the experiment died 16 rats.

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Tissue preparation was performed in two ways:

a) The hippocampus was removed from the brain of the models and was frozen on dry ice, stored at -80℃.

b) For the immunohistochemistry after replacing the blood components the hippocampus was removed from the brain and fixed with a 4% paraformaldehyde.

Western blotting: Protein was extracted from the hippocampus, we dissected 5 mg piece of hippocampus tissue, further added 300 µl ice-cold buffer, homogenized and washed twice with lysing buffer, and then placed in a shaker at 4℃ for 2 hours. Then put to centrifuge at 12,000 rpm at 4°C for 20 min, the samples were separated by 12% sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and later transferred onto a PVDF membrane for the 55 mins, using by electrophoresis. Then membrane was blocked in nonfat milk and kept in water bath at 37º C for the 2 hours. Rinsed one times and incubated with the primary antibody: goat anti DCX polyclonal antibody (1:100, SanYing Biotechnology, China), β – actin (1:1000) for the 12 hours at 4°C. Three times rinsed with Trip-Buffered Saline Tween-20 (TBS-T) for 10 mins. The goat anti-rabbit IgG secondary antibody was diluted 2:2000, the bands placed in water bath for 2 hours with secondary diluted antibody at 37°C. Were washed three times with Trip-Buffered Saline Tween – 20, 10 mins each. The bands visualized using Automated BioSpectrum Imaging system and luminescent liquids (BeyoECL Plus A POO18-1, BeyoECL Plus B POO18-2, at a 1:1 ratio). The gel strip images were obtained. Image analysis used to calculate the optical density of each band, to obtain the expression in the tissue protein.

Preparing and homogenizing the samples: The tissues were homogenized using the TRIzol® Reagent, Life Technologies Corporation, USA; license №358

Expressions miR-134 by qPCR in the Hippocampi of mature epileptic rats: Homogenization of the hippocampus produced with using laboratory-blender, after grinding added to RIPA buffer, performed centrifugation 12,000 rpm, 4C for 10 minutes, saved the liquid. Titration of microRNA-134-5p in solution showed that this microRNA has a high concentration. The concentration of microRNA-134 was tested using the spectrophotometer UV 1102II, where we have used the formula: (ABS (1) -ABS (3)) *40’000 to each group. We are used hippocampus of the brain tissue samples from the experimental animals, in order to provide a template for polymerase chain reaction (PCR) amplification.Prepare the reaction mixture by combining the indicated volumes: 2xTrans, passive dye ddH2O, primer F, primer R and specific primers. To purify the PCR products were used TRIzol® Reagent (Life Technologies Corporation, USA).qPCR reaction was performed by 7500 Real Time PCR System v2.0.6 (Life technologies™; Instrument Serial No. 215007817), according to the manufacturer’s instructions (Life Technologies Corporation, USA). Bulge-Loop™ miRNA qRT-PCR Primer (Riobio Co., LTD;Lot no. R100315), Bulge-Loop™ rno-miR-134-5p, Forward primer (Riobio Co., LTD;Lot: ssD809230833), Bulge-Loop™miR-Reverse Primer (Riobio Co., LTD;Lot no. ssD089261711), Bulge-Loop™ rno-miR-134-5p RT primer, (Riobio Co., LTD;Lot no.ssD809230141)from tissue samples of animal. cDNA synthesis includes two mixes. qPCR was performed in triplicates. The expression level of microRNA was calculated using the comparative CT method. The expression of U6 was used as internal control.

Immunohistochemical analysis of neurogenesis marker-neuronal migration protein DCX: The material after fixation washed with H2O for 6 hours/overnight. Followed 70%, 80%, 90%, 95% the ethanol was evaporated for 1 h, and then 100% for 30 min. Then used transparent Agent: alcohol=1: 1 and processed for 30 min and then in xylene for 5 minutes until transparent. Placed in xylol: paraffin=2: 1, xylol: paraffin=1: 1 mixed solution of each procedure were 30 min, and then placed in paraffin wax for 1 hour. Pieces of tissues carefully poured in the melted wax, quickly immersed in water. A small piece, placed on a glass slide, added a drop of water. Then she took a small piece of wax placed in a 30% ethanol solution on the surface. The material put in warm water 40-45℃, and then was baked at 60℃ oven for 2 hours until the wax melts.

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Immunohistochemistry: Crushed paraffin slice was washed with xylol for 10 min, the glass was placed in alcohol: xylene=1: 1, 100%, 95%, 90%, 80%, 70%, each procedure was performed for 5 min with distilled water, washed 3 times. Then were incubated in 3% H2O2 at room temperature for 20 min (in the dark), washed in PBS 3 times. We placed at room temperature in normal bovine serum albumin solution in a closed condition. After 30 minutes removed the serum and added anti-working solution 1:100 and incubated for 37℃ for 5 hours or 4℃ overnight. Washed in PBS 3 times, each time 5 minutes. Added auxiliary agent polymer, and incubated at 37℃ for 30 min, then washed in PBS 3 times, each time 5 minutes. Added horseradish peroxidase-labeled goat anti-rabbit or goat anti-mouse IgG, incubated for 37℃ 30 minutes, with PBS washed 3 times, each time 5 minutes. Added the color liquid for 8 min, rinsed. Staining with hematoxylin 4 min, rinsed with distilled water 3 times, and placed in hydrochloric acid 3 times. Then used xylene, each process lasted 2min. The glass is placed in a hood to dry. Used the microscope and camera.

Statistical analysis: Results were analyzed by oneway ANOVA Turkey's multiple comparison test and the value of CT miR-134 was determined using Graphpad analytical software.

Results:

Expression of neuronal migration protein doublecortin.

Neuronal migration protein - DCX was isolated from rat brain (hippocampus) and was observed in a 40kDa power dissipation. Western blotting showed that this protein - DCX was expressed in epileptic groups while in control group there had been a dramatic decreasing as shown in Fig. 1. Band the endogenous control b-actin remained constant.

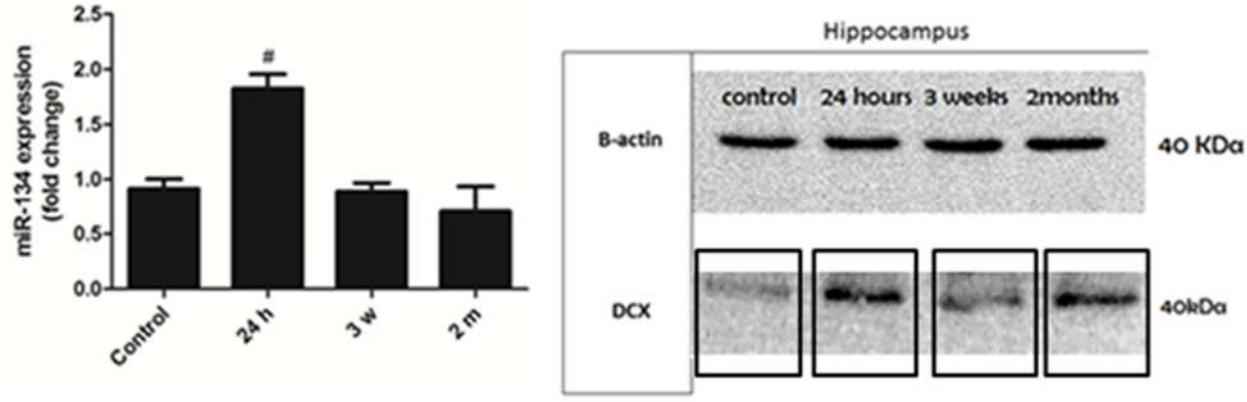

Fig.1: Protein expression analysis with western blotting. β-actin with absolute integrated OD of each band: Line1-1175.4, Line2-1371.5, Line3-1553, Line4-1559.1and. Blots of crude rat brain extract of DCX. Line1 (control group), Line2 (24hours), Line3 (3 weeks), Line4 (2months). In bar graph depicting of extraction of MicroRNA-134 in all animal groups by qPCR. Values are indicated as means± SEM. #P< 0.05 compared with controls.

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Expression of miR-134-5p using qPCR: The results of the experiments on animal model using by qPCR, here, the CT value of microRNA-134 in control group reached the level - 0.6-1.1, in the group of 24 hour in acute period of epilepsy shows the expression of microRNA-134 (CT value 0.3-2.6) which had sharply increased, but after 24 hours the expression of microRNA-134 begins to decrease. The CT value of microRNA-134 in 3 weeks - 0.3-1.2, in 2 months 0.3-1.4 (shown in table 1). This finding shows the content of seizures associated with MicroRNA-134 in the hippocampus of rats, which was increased in 24 hours and remained high up to one week inclusive, after 2 months there has been a gradual decrease in expression of microRNA-134.

Table 1. CT Values of MicroRNA-134 in the rat hippocampus of each group.

Groups	Control	24hours	3weeks	2months
MicroRNA-134	0.6-1.1	0.3-2.6	0.3-1.1	0.3-0.8

Table 2. The concentration ΔCT and ΔΔCT of MicroRNA-134-5p using by qPCR

MicroRNA	Group	ΔCT	ΔΔCT
miR-134-5p	Control	10,09971809	1,589613279
	24h	10,87203884	0,135387739
	3 weeks	12,6053257	1,868674596
	2 moths	11,05960846	0,639157613

Immunohistochemistry:

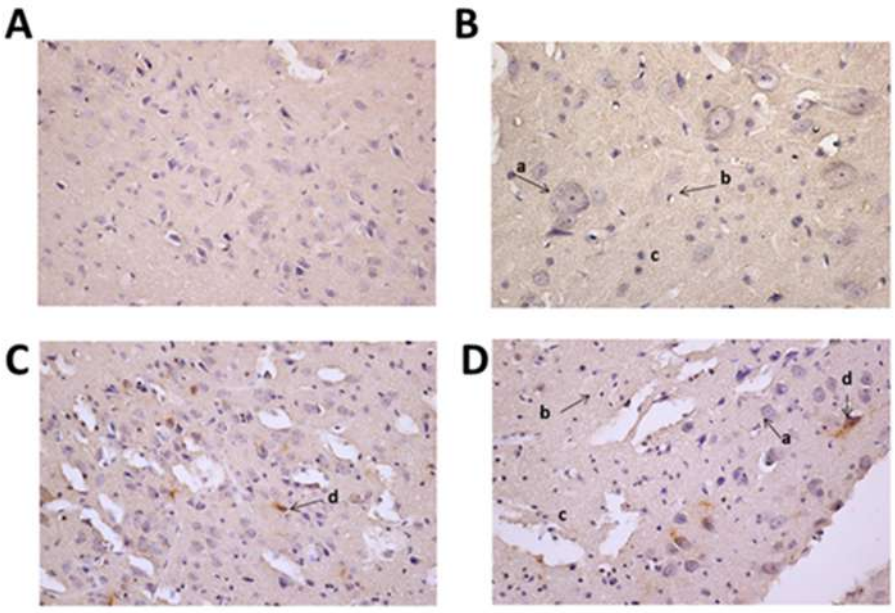

Figure 2: A (Obj x 40) - B (Obj x 60) control groups, C (Obj x 40) –D (Obj x 60) 24 hours after induction of epilepsy in rats. a - neuronal cell, b – glial cells, c – neuropil, d – doublecortin. Hematoxylin and eosin staining.

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Immunohistochemistry: Hematoxylin & eosin stained hippocampus sections show that the control group (A, B) has a different histological pattern (structure) than epileptic pilocarpine-induced rats (C, D, E, F, G, I). In histopathology pictures we can clearly see the neuronal cells (a) across the glass surface, also visible are isolated glial cells (b), the accumulation processes of nerve cells - neuropil (c), which is the normal condition in the hippocampus of a mammal. Doublecortin identified by immunohistochemical staining of the brain sections of animal models with epilepsy, in some cells of the granular and molecular layers were observed neuronal migration protein – doublecortin, which was localized in the cytoplasm of cells, but not in their kernel. Group of 24h (C, D) after induction of epilepsy has been observed pattern with a single positive cells (red-burgundy color) - doublecortin (d).

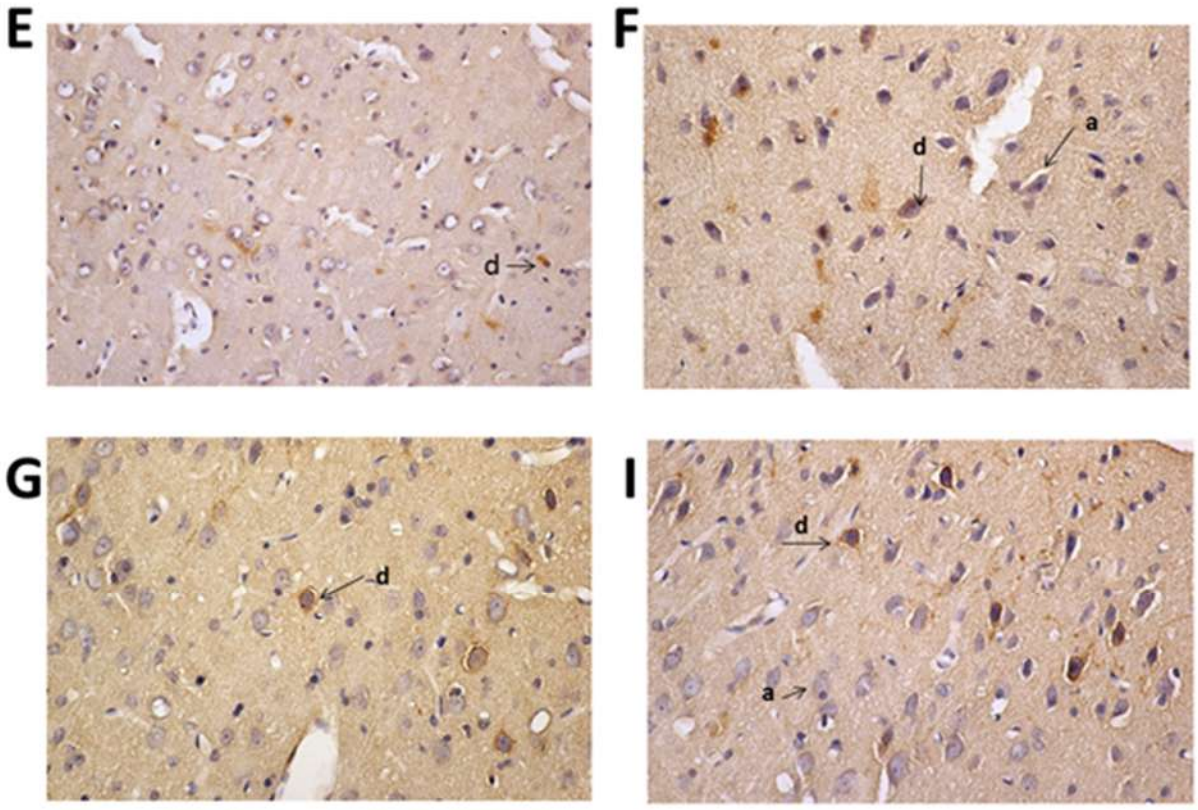

Figure 3: E (Obj x 60) - F (Obj x 60) 3 weeks, G (Obj x 60) – I (Obj x 60) 2 months after induction of epilepsy. a - neuronal cell, b – glial cells, c – neuropil, d – doublecortin.

In group 3 weeks (E, F) increasing the numbers of neuronal migration protein – doublecortin (d). The fourth group - 2 months (G, I) there was a sharp increase in the number of neuronal migration protein - doublecortin.

These figures can show us clearly the increase in the number of neuronal protein - DCX (d), thus, this protein may participate in neurogenesis in epilepsy.

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Discussion:

The process of constant formation of new cells in the hippocampus of adult mammals was first discovered by Altman et al. in the 60-ies of XX century. Over the fifty years that have passed since then, it has been established that some of these cells become neurons and integrate into existing network of the hippocampus, a part gives rise to glia. The formation of new cells and their differentiation into neurons or glia is under the regulatory influence of different external and internal factors. One of these factors is seizure activity. An assumption was made about the contribution of postnatal neurogenesis in the development of convulsive states, which stimulated research in this area.

Neuronal migration protein (DCX) can be reduced by enhancing cell migration and whether such repairs can reduce the risk of seizures (Jean-Bernard Manent., 2009). In adults, DCX has been employed as a reliable marker for new neurons in regions, such as the dentate gyrus, where ongoing neurogenesis is recognized (Arvidsson et al., 2002; Brown et al., 2003; Couillard-Despres et al., 2005). The experiments on rats show the marker changes in the level of MicroRNA-134 and DCX at different time periods after seizures. A large amount of microRNA-134 leads to a reduction of neuronal activity and may help to inhibit the degree of activation of neurogenesis. A microRNA-134 level in the hippocampus of epileptic rats begins to decline after three weeks. qPCR results of MicroRNA-134 values of CT have shown: After 3 cycles of PCR amplification, MicroRNA-134 CT values (shown in table 1) of 24-hour group had a significant difference compared with the control group, expression was increased in the direction of 24 hours, thus leads to a decrease in the activity of neurons and thus can help to reduce uncontrolled movement. In other groups the expression level of MicroRNA-134 three weeks began to decrease. In comparison with the control group the latter two groups (3 weeks and 2-month group) had a significant decrease in the expression of microRNA-134. Quantification of the number of MicroRNA-134 demonstrated an average of new neurons in epileptic hippocampus. The results have shown that after epileptic seizures content with MicroRNA-134 in the hippocampus of rats is reduced in three week, and after 2 months, which is quite possibly due to the influence of pilocarpine-inhibition of MicroRNA-134 where a decrease in the inhibition of synaptic plasticity (Yan Zhu et al., 2015), which corresponds to the data of the previous experiments to Yang Xiaolan and Yan Zhu et al. According to their experiments the level of expression is associated with pilocarpine-inhibition of MicroRNA-134 and a decrease in the inhibition of synaptic plasticity. Interestingly, the overall decline of neurogenesis was significantly higher in rats, increased frequency of seizures in chronic epilepsy more disastrous for dentate neurogenesis. Western blot experiments showed that expression of DCX is determined at the level of the hippocampus in epileptic groups. The differences compared to twenty-four hours, the levels of three weeks and two months (eight weeks) groups were significantly increased (shown in figure 1). Each model of epilepsy in animals is complex due to its cellular and molecular variability. Immunohistochemistry study showed that neuronal migration of protein – doublecortin, begins to be expressed after the induction of epilepsy, that is confirms that this protein is part of neurogenesis. In a number of studies have shown that seizures caused by administration of chemoconvulsants or electric irritation profileproven parts of the brain, accompanied by increased cell proliferation in the hippocampus and an increase in the number of young neurons in it. With that there are proteins present only in immature, young neurons, which allows the level of their expression directly to determine the intensity of neurogenesis. One of these markers is neuronal migration protein – doublecortin, which is a protein associated with immature neurons and mediating the processes of their migration. Doublecortin is expressed in neuroblasts and immature neurons for several weeks after their formation. The process of maturation of the neuron, its incorporation into the granular layer of the dentate fascia, the branching of dendrites and establish synaptic connections with pyramidal cells of field CA3 is accompanied by the gradual disappearance of the expression of doublecortin. Mature neurons in the granular layer of the dentate fascia already does not Express doublecortin. In this regard, doublecortin can be used as a marker of immature neurons that will allow us to study the processes of postnatal neurogenesis. We have shown in our experiment that the neuronal migration protein - DCX is produced in epileptic experimental models. Epilepsy damages the hippocampus. In the chronic

stage of epilepsy in adults occurs hippocampal sclerosis, which may reduce the growth of new neurons. Under normal conditions, the concentration of microRNA-134 can effectively inhibit the abnormal formation of synapses. Researchers have found microRNA-134 in particularly low levels in areas of the brain associated with epileptic seizure, especially in the hippocampus. Detailed knowledge of regulation in the MicroRNA-134 and DCX are not fully understood and requires further experiments and new approaches to the study of signaling pathways (Yan Z., 2015).

The results offer hope for people suffering from epilepsy, which there is no benefit from current medications. It is likely that this drug-resistant group of patients could significantly benefit from a new approach. This study can also help us to learn more about the signaling pathway of MicroRNA-134 and DCX. Grasping the significance of the pathogenesis of epilepsy may contribute to its further in-depth study, at a genetic-level. These may provide a new idea for the gene therapy of epilepsy. The new research is not yet transferable to humans, research needs to be work to the end to be sure that future discoveries in the treatment of epilepsy, where the role of microRNA in epilepsy is considered to further treatment. In experimental reviews the chronic models of epilepsy in human epileptic tissue, showed the results suggesting a reduction in hippocampal neurogenesis in late stages of the disease (Mikkonen M et al., 1998; Liu Y. W, et al., 2008). Investigating the expression of miRNA-134 and DCX in epileptic animal model can provide us with the understanding of the molecular mechanisms underlying epileptic tolerance and may help identify novel neuroprotective strategies to protect against to epilepsy disorder.

Conclusions: Our study showed that epilepsy has been accompanied by the increased levels of microRNA-134 to 24 hours inclusive, after 3 weeks the expression of microRNA-134 began to decrease. Research had shown an increase of neuronal migration protein – doublecortin in the hippocampus in epilepsy models, these changes in the hippocampus is results suggest the synaptogenesis. Immunohistochemical analyses showed that the numbers of neuronal migration protein - DCX after inducing epilepsy, we assume that the neuronal migration protein - DCX is involved in neurogenesis.

Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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