Dynamic structural neuroplasticity during and after epileptogenesis in a pilocarpine rat model of epilepsy

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Abstract

Background: The role of neuroplasticity in epilepsy has been widely studied in experimental models and human brain samples. However, the results are contradictory and it remains unclear if neuroplasticity is more related to the cause or the consequence of epileptic seizures. Clarifying this issue can provide insights into epilepsy therapies that target the disease mechanism and etiology rather than symptoms. Therefore, this study was aimed to investigate the dynamic changes of structural plasticity in a pilocarpine rat model of epilepsy.

Methods: A single acute dose of pilocarpine (380 mg/kg, i.p.) was injected into adult male Wistar rats to induce status epilepticus (SE). Animal behavior was monitored for 2 h. Immunohistochemical staining was performed to evaluate neurogenesis in the CA3 and dentate gyrus (DG) regions of hippocampus using biomarkers Ki67 and doublecortin (DCX). The Golgi-Cox method was performed to analyze dendritic length and complexity. All experiments were performed in control rats (baseline), at 24 h after SE, on day 20 after SE (latent phase), after the first and 10th spontaneous recurrent seizures (SRS; chronic phase), and in non-epileptic rats (which did not manifest SRS 36 days after pilocarpine injection).

Results: SE significantly increased the number of Ki67 and DCX-positive cells, suggesting neurogenesis during the latent phase. The dendritic complexity monitoring showed that plasticity was altered differently during epilepsy and epileptogenesis, suggesting that the two processes are completely separate at molecular and physiological levels. The numbers of spines and mushroom-type spines were increased in the latent phase. However, the dendritogenesis and spine numbers did not increase in rats that were unable to manifest spontaneous seizures after SE.

Conclusion: All parameters of structural plasticity that increase during epileptogenesis, are reduced by spontaneous seizure occurrence, which suggests that the development of epilepsy involves maladaptive plastic changes. Therefore, the maladaptive plasticity biomarkers can be used to predict epilepsy before development of SRS in the cases of serious brain injury.

Keywords: Epileptogenesis, Neuroplasticity, Spine numbers, Dendritic complexity, Neurogenesis
Background
Epilepsy is a chronic neurological disorder characterized by spontaneous recurrent seizures (SRS), which develop gradually during a process called epileptogenesis [1]. The molecular events underlying the conversion of a normal to an epileptic brain are not completely understood yet [2]. Acquired brain pathology such as tumor, infecition, stroke and traumatic brain injury (TBI) causes epilepsy after an unpredictable latent period [3]. Acute neural damage can also result in SRS in animal models after an unlimited latent phase [4]. Epilepsy development is typically a three-phase process in humans and animal models: first, the occurrence of precipitating damage or events; second, a latent period which consists of molecular events that mediate the transformation of a normal to an epileptic brain (epileptogenesis); and third, chronically established epilepsy characterized by SRS [5]. Previous studies have confirmed that seizure incidence and epileptogenesis are two distinct events because anticonvulsants which can terminate an ongoing seizure or prevent the occurrence of future seizures in epileptic patients are ineffective in blocking epileptogenesis [6].

Previous studies have revealed that SRS causes neuronal death in brain tissues, so epilepsy could be considered as a neurodegenerative disease [7]; however, increasing lines of evidence have confirmed the involvement of neurogenesis and neural plasticity in epileptic patients [8]. The ability of the central nervous system (CNS) to restructure itself in response to internal and external stimuli is an important feature that contributes to neural adaptation to unhealthy conditions especially epilepsy [9]. According to the previous studies, incidence of a single seizure could trigger plastic changes in the dentate gyrus (DG) [10, 11]. During the epileptogenesis process after neural damage, a series of events including neurogenesis, plasticity changes and neural network remodeling is induced possibly through organization of new circuits, dendrite and axon growth, and restructuring spines, which have important implications in hyper-synchronization during SRS [12, 13]. Therefore, the neuronal damage accumulates in the CNS until reaching a threshold for an unprovoked seizure [3, 5]. Considering the unpredictable and unlimited timeline from CNS damage to epilepsy in humans, most studies in this area are focused on neuroplasticity changes in epilepsy rather than in epileptogenesis [14, 15]. Epileptogenesis describes the process of molecular and structural modifications and neural network remodeling leading to seizure activity in a normal brain. The main area that is involved in the initiation, development and termination of epileptic seizures is the hippocampus, which is a unique area capable of producing new neurons in adult brain [16, 17]. However, it is still unknown whether plasticity changes in hippocampus are a primary cause or a consequence of epilepsy. In addition, determining which kind of plasticity (adaptive or maladaptive) occurs during epileptogenesis may provide insight into novel treatment strategies for epilepsy. As neurogenesis and dendritic spines are important components of structural plasticity [18], in this study, we set out to dynamically investigate neuroplasticity by using neurogenesis and neurodevelopmental biomarkers (Ki67 and doublecortin [DCX]) [19], and arborization patterns during and after epileptogenesis in a pilocarpine rat model of epilepsy, which is the most prevalent model for temporal lobe epilepsy (TLE).

Material and methods
Animals
Adult male Wistar rats weighing 250–300 g with average age of 3–5 months were maintained at 20–25°C with a 12-h light–dark cycle and free access to compressed animal feeds and municipal tap water. This study was approved by the Ethics Committee of University of Tabriz (Tabriz, Iran) which was reconciled to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Animal model of epilepsy and experimental design
The pilocarpine rat model of epilepsy is one of the most prevalent models of human TLE, which exhibits physiological, behavioral, electroencephalographic and seizure patterns resembling those of TLE. Therefore, this model is widely used in status epilepticus (SE) and epileptogenesis studies [4]. Thirty minutes prior to intraperitoneal (i.p.) injection of pilocarpine, animals (n = 60) received a subcutaneous scopolamine methyl nitrate (1 mg/kg) to reduce peripheral cholinergic effects of pilocarpine. Then, a single acute dose of pilocarpine (380 mg/kg, i.p.) was injected to induce SE. Animal behavior was monitored for 2 h after pilocarpine injection and Racine’s scale was used to evaluate pilocarpine-induced seizures [20]. The rats gradually showed different stages of seizures. These stages were evaluated according to the Racine’s scale. Stage 0: without any response; stage 1: mouthing and facial movement; stage 2: head nodding; stage 3: forelimb clonus; stage 4: rearing with forelimb clonus; and stage 5: rearing and falling with forelimb clonus. Continuous seizure activity (stages 3–5) within 2 h was considered as SE or acute phase, which was terminated after 2 h by diazepam (10 mg/kg, i.p.; Sigma-Aldrich). Following the pilocarpine treatment, all rats were observed for development of SRS for 5 days/week and 8 h/day (at least 40 h per week). Spontaneous seizures (stages 3–5) occurred 25 ± 5 days after pilocarpine injection.

To dynamically evaluate the structural neuroplasticity events, animals were sampled and observed at 5 time points as follows (Fig. 1): (1) 24 h after SE (n = 8), a time...
Day 20 after SE (n = 8), in latent phase; (3) 24 h after first SRS (n = 8), which indicates the beginning of chronic phase; (4) after 10 times of SRS (n = 8), the rats exhibited an average of 5 ± 2 spontaneous seizures/week); (5) 36 days after SE without SRS (non-epileptic rats, n = 8), the rats experienced SE but did not manifest SRS 36 days after pilocarpine injection. An additional group of control rats (baseline, n = 8) were injected with scopolamine methyl nitrate, diazepam and saline instead of pilocarpine to obtain the equivalent shock from scopolamine methyl nitrate, pilocarpine and diazepam injections.

Immunohistochemistry
Ki67 and DCX protein expression was assessed in CA3 and DG regions of hippocampus by immunohistochemistry using anti-Ki67 and anti-DCX specific antibodies. Ki67 is a cell cycle protein marker closely associated with cell proliferation. The use of Ki-67 as a marker of proliferation in the initial phase of adult neurogenesis has been validated [19]. DCX is a protein that facilitates microtubule polymerization and is expressed in migrating neuroblasts and immature neurons, which can be classified as a marker of adult neurogenesis in brain [21]. Consequently, increased Ki67- and DCX-positive neurons can suggest neurogenesis in the DG region of adult rats.

A total of 18 rats, including three from control (baseline) and 2–6 from experimental groups, were used for immunohistochemistry. Rats were sacrificed under deep ketamine and xylazine anesthesia, perfused with 0.3% sodium sulfide in 0.1 M phosphate buffer (PB), followed by 4% formaldehyde in PB for fixation. An additional group of control rats (baseline, n = 8) were injected with scopolamine methyl nitrate, diazepam and saline instead of pilocarpine to obtain the equivalent shock from scopolamine methyl nitrate, pilocarpine and diazepam injections.

Neurons expressing Ki67/DCX was quantified by computerized image analysis. High-quality images were digitalized with a resolution of 768 × 494 pixels and were analyzed using Image J software. Ten fields per section were evaluated and the numbers of Ki67/DCX-positive cells were counted among DG granular cells. The mean density (cell count/mm²) and SD were calculated for each group. The image analysis was performed by an experimenter blinded to the experimental conditions. Data were obtained from three rats.

Golgi-cox staining
To visualize neurons in their entirety including cell soma, axons, dendrites, and spines, Golgi-Cox staining was performed according to the descriptions by Bayram-Weston et al. [22]. Briefly, rats were transcardially perfused with 0.9% of saline for 3 min followed by 1.5% PFA in 0.9% saline for a further 5 min. After perfusion, dissected brains were fixed in 4% PFA for 24 h and impregnated at room temperature for 2 weeks in a mixture of Solutions A, B and C that contained potassium dichromate, potassium chromate and mercuric chloride, respectively, then immersed in 25% sucrose for 48 h or more at 4 ºC. The brain was sectioned into 100-μm slices, incubated in 0.1 M Tris buffer (pH 7.3) and then transferred onto gelatin-coated slides. The slides were dried at room temperature for 24 h, stained with 20% ammonium solution for 10 min and washed with distilled water, followed by gradient dehydration with 70, 95 and 100% ethanol. Afterward, the tissue sections were incubated in xylene twice for 10 min and mounted with DPX. The sections were observed under an Olympus microscope.
BX50 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and analyzed using Neurolucida software (MBF Bioscience, Williston, VT). Dendritic morphology was analyzed by using a 40× objective lens.

Number of spine and spine maturation were evaluated on apical dendrites of CA3 pyramidal neurons and DG granular cells in the ventral hippocampus. Pyramidal neurons were selected based on their cytoarchitecture including a clearly identifiable axon and apical dendrite. Five individual neurons were selected randomly for each animal in all experimental groups and reconstructed by using a computer-based neuron tracing system (NeuroLucida 11.0, MBF Biosciences) with a 40× objective lens. For each neuron, five segments (120–300 μm away from the soma) were analyzed by using a 100× oil immersion lens to obtain the averaged spine density, which was expressed as the number of spines/μm of dendrite. Neurons with the following criteria were analyzed: (1) located in the outer layer of the CA3, (2) separate from neighboring stained cells and (3) complete staining of dendrites which contain visible spines. Spine density was analyzed by an experimenter blinded to the experimental groups.

**Statistical analysis**

Data analysis was performed using the SPSS10 software. Differences between experimental groups and control (baseline) were analyzed with the Duncan’s multiple range test. $P < 0.05$ was considered as statistically significant.

**Results**

In this study, systemic injection of pilocarpine (380 mg/kg) induced SE in more than 90% of the rats. Within 5 min after injection, the rats developed piloerection, diarrhea, and signs of cholinergic stimulation such as tremors in head and body, a well-defined pattern of behavior that was evaluated according to Racine scale (1–5 stages) [20]. Rats that developed stages 3–5 of seizure within 2 h after pilocarpine injection were considered to have undergone SE. About 15% of rats developing SE died due to the harsh damage. The acute phase was terminated by the injection of diazepam (10 mg/kg), followed by a latent phase of 25 ± 5 days. About 80% of the surviving rats developed SRSs after the latent phase, thus entering the chronic phase. Rats that failed to develop SRS after 36 days were considered to be non-epileptic.

**Ki67-positive cells increased during the epileptogenesis**

Ki67 is a marker of cell proliferation that increases in the initial phase of adult neurogenesis [19]. Ki67 immunohistochemistry illustrated neuron proliferation from progenitor cells in CA3 and DG (Fig. 2a). The Ki67-labeled cells in hippocampus were significantly increased in the latent phase, compared to the control group.
The Ki67-positive cells in control rats confirmed the occurrence of neurogenesis in DG of adult brain, which is consistent with previous studies [23]. The number of Ki67-positive cells showed a 1.4-folds increase at 24 h after SE, and reached a peak on day 20 after SE (about 2 folds of the control level). Then, the Ki67-positive cells were significantly reduced in the chronic phase compared to the latent phase ($P < 0.05$) (Fig. 2b). Interestingly, the number of Ki67-positive cells on day 36 after SE without SRS (27.1 ± 1.34 cells/mm$^2$) was significantly higher than that of age-matched rats developing SRS (23.1 ± 1.32 cells/mm$^2$) and that of control rats (21.2 ± 1.06 cells/mm$^2$).

**DCX-positive cells increased during epileptogenesis**

The DCX protein binds to microtubules and plays an important role in neuronal migration and differentiation, so it can be used as a biomarker for newborn neuronal development [24]. In this study, DCX expression showed a similar increase pattern as Ki67 in the latent phase, which confirmed neurogenesis and neural development in the latent phase. The number of DCX-positive cells was about 43.2 ± 2.18 cells/mm$^2$ in control rats (baseline), and it was increased to 50.24 ± 2.5 cells/mm$^2$ at 24 h after SE. This increase was milder than that of Ki67-positive cells at the corresponding time point. Then the DCX-positive cells were reduced at 24 h after the first SRS manifestation, a time point indicative of chronic phase initiation. The DCX-positive cells in the SRS chronic phase (after 10 times of SRS) were slightly but not significantly increased compared to the control (baseline, $P > 0.05$). In addition, the number of DCX-positive cells in rats that manifested 10 SRSs (47.8 ± 2.39 cells/mm$^2$) was significantly less than age-matched non-epileptic rats (55.5 ± 2.82 cells/mm$^2$; Fig. 2c).

**Dendritogenesis is increased during the latent phase**

Golgi-Cox staining was used to evaluate changes in dendritic complexity, which can affect neuronal network remodeling and is prerequisite to circuit reorganization. Comparison of dendritic density in the hippocampus before and after SE revealed that continuous seizures induced wide neuronal death in the whole hippocampus especially in CA3 region (Fig. 3). After SE, dendritogenesis increased during the latent phase, but was further inhibited by recurrent seizures especially in CA3 region in the chronic phase. In addition, the dendritic length and complexity increased during the latent period compared to the control group (baseline, Fig. 4). The rats that experienced SRS showed reduced dendritic length depending on the number of SRS episodes: the epileptic rats experiencing 10 times of SRS showed more degeneration of dendritic arbors than those undergoing only one SRS episode. Interestingly, the neuron density of rats that experienced SE but no SRS (non-epileptic rats) was not increased compared to the age-matched rats that experienced SRS (after 10 times of SRS) and to the rats on day 20 after SE. The dendritic length and complexity of non-epileptic rats did not
Spine density in CA3 and DG is increased during epileptogenesis

The dendritic spine density reflects the synaptic plasticity in CA3 region of hippocampus. Spines are small membranous protrusion from a neuron’s dendrite that typically receive input from a single axon and transmit electrical signals to the next neuron, thus playing an important role in circuitry networks [25]. Furthermore, changes in the morphology or number of dendritic spines are strongly implicated in synaptic plasticity, learning, memory and long-term potentiation [26]; thus, abnormalities in dendritic spines could cause cognitive deficits observed in epileptic patients. The spine density was analyzed based on spine morphology [27]. The spine density was 122 ± 7 spines/μm length of dendrite in control rats, and reduced to 90 ± 5 spines/μm length of dendrite at 24 h after SE. However, it was increased during the latent phase and reached a peak at 20 days after SE (201 ± 10 spines/μm length of dendrite), and then reduced afterwards (Fig. 5a). Interestingly, the spine density in non-epileptic rats was 131 ± 8 spines/μm length of dendrite, which did not significantly differ from the control (Fig. 5b). We also quantified the mushroom type of spines as mature spines, which play an important role in plasticity [28]. SE significantly reduced the mushroom type of spines in CA3 (Fig. 5c). The number of mushroom spines was 20 ± 1.5 mushroom spines/μm in control rats, reduced to 11 ± 0.75 mushroom spines/μm at 24 h after SE, and increased to 50 ± 2.8 mushroom spines/μm at 20 days after SE (Fig. 5c). The density of mushroom spines was then decreased significantly after incidence of first SRS ($P < 0.05$), but did not change further at 10 times of SRS. Interestingly, the number of mushroom spines in the non-epileptic rats was 23.5 ± 1.15 spines/μm, which was comparable to that of the control and significantly less than those of the epileptic rats (Fig. 5c).

Discussion

Epilepsy is primarily a disorder of electrical excitability that is characterized by unprovoked recurrent seizures at the physiological state [1]. Seizure starts from hyper-activation of some defective neurons and proceeds with excessive excitability of a large group of surrounding neurons; this uncontrollable procedure is called hyper-synchronization [29]. In a healthy brain, the transfer and the frequency of action potentials are controlled strictly but changes in circuit networks or neurotransmitter release may cause hyper-activated epileptic brain [30]. In common types of epilepsy, the defected neurons located in the temporal lobe and hippocampus play important roles in initiation, development and termination of recurrent seizures [31]. Previous studies have shown an association between neuroplasticity and epilepsy but it remains unclear whether neuronal plasticity is a...
consequence or a prerequisite of epilepsy. Clarifying this question may provide guidance for changing treatment strategies from symptomatic to mechanistic and introduce novel targets for drug design.

In this study, the immunohistochemical results showed that Ki67-positive cells were increased after pilocarpine injection, the increase continued during epileptogenesis and reached a peak at the end of the latent phase, and then the Ki67-positive cells were reduced along with SRS. The neuronal development marker DCX showed a similar pattern of change as Ki67 (Fig. 2). These results are in agreement with previous experiments confirming that seizure acts as a stimulator of neurogenesis [10, 11]. Induction of SE by an acute dose of pilocarpine caused neuronal damage and reduced the amount of hippocampal neurons (Fig. 3). These results, combined with previous studies [32], suggest that CNS injuries trigger neurogenesis as an adaptive response, therefore there is a growing number of neural cells in the latent phase in response to neuronal death rather than seizure itself. The neurogenesis continues during the latent phase, resulting in high numbers of neurons in the hippocampus at 20 days after SE. Therefore, we conclude that continuous seizure activity which induces neurogenesis in SE-experienced rats is different from neurodegeneration that is imposed by epileptic seizures in the chronic phase. Interestingly, the Ki67 and DCX-positive cells in non-epileptic hippocampus are similar to the control, which suggests that neurogenesis is essential in epilepsy development. The seizure-induced newborn neurons have potentials to amend the defected circuits in hippocampus, thus contributing to hyper-synchronization.

Our results also confirmed that the dendritic complexity increases during the latent phase. The non-epileptic group did not show neurogenesis and dendritic complexity compared to the epileptic rats (rats manifesting SRS), indicating that neurogenesis and dendritogenesis are essential for subsequent recurrent seizures. Dendritogenesis promotes new synaptic connections and aberrant circuitries, which may cause hyper-synchronization in epileptic seizure. Therefore, it seems that a threshold of dendritogenesis, circuits remodeling and other

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Fig. 5 Spine density and number of mushroom-type spines in epileptic rats. a Spines observed at 40x magnification in the CA3 region. Scale bar represents 5 μm. b Quantification of spines per μm of dendrites length. The amount of spines increased during the epileptogenesis, and then decreased after 10 times of SRS. c Quantification of mushroom spines. Data are presented as mean ± SEM. * P < 0.05, ** P < 0.01 and *** P < 0.001.
unknown events are required to firing large electrical impulses in millions of neurons in the whole brain that lead to a generalized seizure. Neurogenesis and dendritogenesis could contribute to a compensatory response, as a form of homeostatic plasticity, to excessive neuronal death in the SE phase. Thus, any type of injury or stimulus such as trauma and infection that promotes neurogenesis or dendritogenesis can cause uncontrolled neural network remodeling, which could trigger epileptic seizures [33].

We also examined structural remodeling of synapses by analyzing spine shape alterations during and after the epileptogenesis. Previous results have shown a significant decrease in dendritic spine density in hippocampal pyramidal neurons and DG neurons in patients with TLE [34]. But others have reported a significant increase of spine density in pyramidal neurons of epileptic patients [35]. The elevated risk of seizure has also been documented in other neurological diseases such as Alzheimer’s disease and Huntington’s disease, which are accompanied by dendritic spine pathology [36]. These findings strongly suggest that dendritic spine abnormalities play a central role in the pathophysiology of epilepsy [37]. However, the specific pathogenic role and clinical consequences of this dendritic pathology in epilepsy are not fully understood. It is unclear whether these dendritic abnormalities are more related to the cause or the consequence of epileptic seizures. Our results showed that the dendritic spine abnormalities especially in the CA3 region of hippocampus are related to the cause of epileptic seizures. Continuous seizure activity in the SE phase distracts spines and reduced spine density and the mushroom-type spines in the CA3 area. The spine density was improved late in the latent period which may be attributed to spine generation and the number of mushroom-type spines was also increased which may be attributed to spine maturation. However, during the chronic phase, the number of mushroom-type spines did not change significantly at the first versus 10th SRS episode. In addition, our results showed more spines in the chronic phase than in the acute phase and control (baseline). Pathological studies following seizures induced by pentylenetetrazole or electrical kindling have demonstrated dendritic spine loss and beading of dendrites in the neocortex and hippocampus [38]. Here, comparison of epileptic and non-epileptic hippocampus showed that the spine density and the number of mushroom spines were significantly decreased in nonepileptic rats that did not experience SRS. Our results also confirmed that spontaneous seizure significantly reduced spine density during chronic phase in comparison with latent phase which refers to neurodegeneration, it could be observed in epileptic patients also [39]. Alterations in dendritic spines represent a form of seizure-induced brain injury that might contribute to learning disabilities and other cognitive deficits commonly seen in epileptic patients [38, 39]. Similar to epileptic patients, it is possible that accumulation of neural damage after pilocarpine injection promotes hyperexcitable circuits and neural network or circuit remodeling, which finally cause a tendency toward seizures. Overall, our study demonstrated that significant changes in dendritic length, shape, and branching patterns and also spine density in the latent phase are required for the incidence of epilepsy.

Conclusion
Accumulation of CNS damage stimulates axon growth, restructuring of spines, production of new circuits, and synaptic remodeling, which finally can lead to maladaptive plasticity. Therefore, the structural plasticity is required for SRS in rats that have undergone ES. Our results confirmed that the CNS has different manners of plasticity during epileptogenesis and epilepsy. It is possible to detect maladaptive plasticity biomarkers after serious injuries of the CNS, which can be promising predictors of epilepsy before the occurrence of SRS. Considering the similarities between pilocarpine model of epilepsy and human TLE, the molecular machinery underlying neuroplasticity may be used as candidate targets for epilepsy therapy. However, more studies should be performed before we can make a solid conclusion.

Abbreviations
CNS: Central nervous system; DCX: Doublecortin; DG: Dentate gyrus; SE: Status epilepticus; SRS: Spontaneous recurrent seizure; TBI: Traumatic brain injury; TLE: Temporal lobe epilepsy

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Authors’ contributions
SH performed experiments, analyzed data and wrote the paper. LS contributed substantially to the conception and design of the study, the acquisition of data, and the analysis and interpretation. All authors read and approved the final manuscript.

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Ethics approval and consent to participate
All the experiments were approved by the Ethical Committee of Tabriz University of Medical Science (Tabriz, Iran) and conform to the European Communities Council Directive of 24 November 1986 (86/609/EEC). This manuscript does not contain in vivo human studies.

Consent for publication
All authors have agreed to be listed as contributors and have read and approved the publication of this manuscript.
Competing interests
All the authors have no conflict of interest to declare.

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