EFFECTS OF KINDLING AND EPILEPTIC SEIZURES DURING PREGNANCY ON CEREBELLAR EXPRESSION OF THE PSA-NCAM IN RAT PUPS

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ABSTRACT

Background: Maternal epileptic seizures during pregnancy could be affect offspring cerebellar function. The polysialylated neural cell adhesion molecule (PSA-NCAM), which is highly expressed in the developing central nervous system, may play important roles in myelination, cell migration, neurogenesis and synaptogenesis. This study to determine the effect of kindling and epileptic seizures during pregnancy on the PSA-NCAM expression in the neonatal rat cerebellum.

Methods: 40 adults' female Wistar rats were randomly divided into five groups: A) Kindle+/Seizure+; pregnant kindled rats that received repeated intraperitoneal pentylen tetrazol, PTZ injections on gestational days (GD) 15, 17 and 19; B) Kindle+/Seizure--; expectant kindled rats that did not receive any PTZ injections; C) Kindle−/Seizure+; gravid non-kindled rats that received PTZ injections on GD15, 17and 19 and D) Kindle−/Seizure−; as the sham controls that received saline with equal volume of PTZ on GD15, 17 and 19, E)Normal pregnant;

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without any interference. Pups randomly selected on post-natal days 1 and 14 (PD1 and PD14) and PSA-NCAM expression in neonates’ cerebellum was analyzed by western blots and immunohistochemistry.

**Results:** Our data showed a significant down regulation of PSA-NCAM expression in the offspring cerebellum of Kindle+/Seizure+ (p=0.001) and Kindle−/Seizure+ (p=0.001) at PD1 and Kindle+/Seizure+ (p=0.01) and Kindle+/Seizure+ (p=0.01) at PD14 groups compared against the sham control and normal groups.

**Conclusion:** Our results demonstrated that during pregnancy, maternal seizures effects on the PSA-NCAM expression in the cerebellum newborns. Probably there is relationship between seizure's pregnancy, PSA-NCAM expression and offspring cerebellar structure or function from epileptic mothers.

**Keywords:** Maternal Seizure, Polysialylated Neural Cell Adhesion Molecule, Kindling, Rat, Cerebellum

1. **BACKGROUND**

Epileptic seizure is a temporary disturbance in brain activity caused by abnormal discharges at the same time is the cerebral cortex (1, 2). Epilepsy is second the most common disorder of the brain after stroke is introduced (3). Epileptic mother's newborns are at greater risk of defects and diseases. Structural changes in neurons through expression of cytoskeletal proteins and neural cell adhesion molecules (NCAM) are done (4, 5). One of the molecules that in recent year's special attention in this regard are that PSA-NCAM is. Previous studies have noted that PSA-NCAM molecules in different brain regions, including cortical, hippocampal formation, the sub ventricular (SVZ) and the cerebellum during development in large quantities and after birth, and even the smaller value is expressed in adolescence, It also indicates that the level of its expression is increased in several cases such as chronic stress, ischemic lesions, brain trauma and epilepsy increases (12, 13). PSA-NCAM molecules as well prevent the destruction of neurons (neuro degeneration) affecting the central nervous system (14). The cerebellum is the part of the CNS plays an important role in motor control. Cerebellum is not starter, but is highly effective in coordinating fine movements a lot. Different neural pathways through the cerebellum and motor cortex and other areas within the brain are starting movements. The cerebellum in puts from sensory systems and other parts, such as spinal cord receives. Therefore, although the damage to the cerebellum is not
paralyzed, but disturbance in fine motor, balance and motor learning even (Motor Learning) is created. In the past, it was thought that epileptic seizures are a phenomenon cerebrocortical, yet there are reports stating that the cortical seizures cause cerebellar damage. Previous studies have shown that for patients with epileptic seizures, serious damage to the white matter of the cerebellum into the relationship between the cerebellum and other areas of the cerebral cortex is faced with disorder (16). Cerebellar atrophy is also a neuropathologic disorder that in some patients with epilepsy is there. Actually, cerebellar atrophy is related with multiple complications such as: severe reduction in Purkinje and granular cells and also gliosis. Although some researchers believe that this injuries not related to epilepsy, but most believe that a direct relationship between epileptic seizures and cerebellar damages is there (17). Previous studies have suggested the cerebellum is very sensitive to hypoxia. Among these structures, including the Purkinje neurons cell body and dendritic trees from other areas in the cerebellar granular and also seem more vulnerable (10). Since PSA-NCAM as a marker of neurons in the CNS is known evolution and migration, and considering the important role of the cerebellum in establishing communications with other parts to the brain such as the cortex. In this study the expression of PSA-NCAM in newborns of mothers with epilepsy to be surveyed. It is hoped that these results will lead in the future to expand the knowledge of embryology and a neuroanatomy relationship between mothers with epilepsy and development in neonates born from these mothers.

2. METHODS
This experimental study was done according to the ethics committee guidelines during 2015 in Mashhad University of Medical Sciences, including the National Institutes of Health (NIH) and protocols of all experiments on animals were approved by the Committee on Animal Care Institute.

3. ANIMALS
For this study, 40 adult female Wistar rats (8 week-old, weighing 165–180 g) were obtained from animal house school of medicine at Mashhad University, Iran. Before the experiment began, to adapt to the environment, all animals with free access to food and water for a week were maintained in normal controlled conditions (12 hr. light-and-dark cycles, at 21 °C with 50% relative humidity). Before mating, the female rats were randomly divided among three groups: Kindled, Non-Kindled (N = 16 in each) and normal group (N = 8).
4. KINDLING PROCEDURE
For kindling, after the adjustment period, the number of females (n = 16), received PTZ with intraperitoneal injection (Sigma, America) at a dose of 40 mg/kg dissolved in one ml of normal saline, every 48 hr. Each rat received total of 12-15 doses of PTZ. After PTZ-injection the convulsive behavior was evaluated for 20 min. Behavior in this model of epilepsy, based upon previous research and Racine's criteria (26) were divided into six phases: stage 0: any response, stage 1: facial and ear's muscles contraction, stage 2: wave of contraction throughout the body, stage 3: jump myoclonic, especially in the upper limbs, stage 4: jumping with myoclonic stand on two legs and fall to the side, stage 5: tonic and clonic falling into the public attacks. To check the maintenance of kindling state, the animals were challenged with a sub-convulsive PTZ dose (40 mg/kg) 10 days after the last kindling injection (13). Kindled (Kindle⁺) rats who are showed generalized tonic–clonic seizures behavior. Non-kindled (Kindle⁻) rats that are no received PTZ-injection before pregnancy, and sham controls (Kindle⁺/Seizure⁻) rats that are received normal saline injected with an equal volumes of PTZ, and normal group without any interference.

5. PREGNANT PROTOCOL AND STUDY GROUPS
14 days after kindling confirmation, every 2-3 kindle or non-kindle rats with male rats were placed in the late afternoon (6-7 PM) in separate cages for mating. In the next morning, vaginal smear were prepared and evaluated with an optical microscope by 100X magnification, if spermatozoa were observed as the 0 day of pregnancy is considered to be. After confirming pregnancy, pregnant rats were divided randomly into 5 groups as follows (N= 8 for each group): Group A: pregnant kindled rats that received intraperitoneal PTZ injections (40 mg/kg) during pregnancy on Gestational Days (GD) 15, 17 and 19 (Kindle⁺/Seizure⁺); Group B: pregnant kindled rats that no received PTZ-injection during pregnancy (Kind⁺/Seizure⁻); Group C: pregnant non kindled rats that received intraperitoneal PTZ injections (40 mg/kg) on GD 15, 17 and 19, exhibiting generalized tonic–clonic seizures (Kind⁻/Seizure⁺); Group D: pregnant non kindled rats that received saline with an equal volume of PTZ, during pregnancy on GD 15, 17 and 19 (the Sham control= Kindle⁻/Seizure⁻) and Group E: pregnant rats without any interference.
6. WESTERN BLOTTING

Rat offspring on PDs 1 and 14 were anesthetized and their cerebellum (N = 4 for each group) were removed following craniotomy. The cerebellums were separated carefully and kept at -70°C. In the next stage; the cerebellar tissues collected were homogenized with lysis buffer (0.3027 gr Tris-Hcl 50mM, 0.4383 gr NaCl 137mM, 0.5ml Triton 100x, 0.0186 gr EDTA 1mM, pH 7.4) containing protease inhibitor cocktail tablet (Roche, Germany), 50 mg of the experimental rats cerebellar tissue with one ml of lysis buffer centrifuged for 20 minutes at 12,300 rpm at 4°C to separate out the cellular debris from the proteins. Cerebellar protein lysates were exposed to SDS-PAGE (10% SDS-polyacrylamide gel) to resolve the proteins approximately 60 minutes at 140V in running buffer (3 gr Tris 20mM, 14.4 gr Glycine 192mM, 1gr SDS solved in 1 Lit DW, pH 7.4). Then by using a Bio-Rad trans-blot apparatus transferred the protein mixture to a PVDF membrane (Millipore, Bedford, MA) for 15 minutes at 300 mA. Membranes were blocked with 5% non-fat milk in TBST (1.22 gr Tris 20mM, 4 gr NaCl 137mM in 500 ml DW containing 1% Tween 20, pH 7.4) for 12 hr at 4°C. Membranes were then washed with TBS-T, incubated with primary monoclonal antibodies (anti-PSA-NCAM (prepared from mouse IgM), diluted 1:5000 in TBST with 5% non-fat dry milk) at 4°C overnight. After 3 washes with TBS-T for remove of excess primary antibody, the secondary antibody a goat anti-mouse IgM diluted 1:10,000 were incubated with membranes in TBST with 5% non-fat dry milk for 2 hr at room temperature. Excess secondary antibody was removed by 3 times (for 5 min each) washing the membranes with TBS-T. Membranes were exposed to ECL substrate for 3 minutes at room temperature. At least, the bands were visible and recorded with the KODAK 1D 3.5.2 image analyzer software (Syngene, UK), and then the band density quantification was demonstrated by using of normalization to the respective β-actin band density (14).

7. IMMUNOHISTOCHEMISTRY

Via inhalation of ether rat pups (N = 4 for each group) were anesthetized on PD1 and 14. The cerebellums were removed immediately, washed in normal saline followed by 4% paraformaldehyde in 0.1 M PBS (phosphate buffered saline), then cerebellums were fixed in normalized fixative consisting 1% paraformaldehyde solution (pH= 7.4) for 48 hr. After tissue processing, embedded in paraffin. The paraffin blocks were cut into 5 μm thickness coronal serial sections. Next, ten Poly-L-lysine slides from paraffin blocks including cerebellum
tissue from each experimental animal were prepared randomly. For de-waxed sections placed in xylene, hydrated through a descending graded ethanol series, and washed in 0.1 M PBS for 10 min. Antigen retrieval was done by the EDTA (pH= 8.4) in PBS at 37 °C for 15 min. Endogenous peroxide activity was quenched by 1% H2O2 in methanol solution in the dark for 30 min. After washing whit 0.05 M PBS plus 0.025% Triton X-100 for 3 times (each for 5 min), PBS plus 10% normal Goat serum with 1% BSA was used to block the tissue for 2 hours at room temperature. Then for decrease the staining background, all the sections were exposed with PBS plus 10% normal goat serum for 30 min. The sections were incubated with primary monoclonal antibody (anti- PSA-NCAM mouse IgM diluted 1:300 in PBS with 1% BSA) in humidified chamber at 4°C overnight. Excess primary antibody was removed by 3 times washed (each for 5 min) with PBS containing 0.025% Triton X-100. Then, the secondary antibody (HRP-conjugated Goat anti-mouse IgM, Vector Laboratories, CA) was added that diluted to 1:500 in PBS with 1% BSA for 2 hr at room temperature. Next, after rinsed with PBS treated with DAB solution (30 mg DAB and 200 μl H2O2 in 100 ml PBS) for 20 min at room temperature in dark. All the sections were counterstained with Harris Hematoxylin for 2 min. Finally, the sections were dehydrated in increasing graded ethanol, deaired in xylene and mounted by cover slip. At last, imaging was performed using by light microscope (Olympus DP12, Japan). For counting the number of PSA-NCAM positive cell Image J application used and data were placed in the formula: $N_A = \frac{\sum \theta}{a/g \Sigma r}$

**Statistical analysis:**
The data of western blot are reported as the relative densities expression of PSA-NCAM, Mean ±SEM. Also the immunohistochemistry data are reported as the number of PSA-NCAM positive cell, Mean ±SEM. To compare differences between groups, one-way analysis of the variance (ANOVA), Tukey post hoc statistical tests and SPSS 22 statistical software were used. A significant difference was defined as p<0.05.

**8. RESULTS**
In this research, both pregnant rat groups (Kindle+/Seizure+ and Kindle+)/Seizure+) received 3 PTZ-injections during GD15, 17 and 19. The cerebellum development in rats initiates from embryonic day 13 (E13), and E14-E20 is a climacteric period for neural migration and neurogenesis in the cerebellum (12). Therefore, we choose GD15-19 for PTZ injections. Cerebellum normally development until 14 days after birth in rat pups, so we study the effect
of kindling and epileptic seizures during pregnancy on cerebellar expression of PSA-NCAM in rat pups on PDs 1 and 14.

**PTZ injection**

During at kindling by intraperitoneally injection of PTZ, in the first injection, rats mild symptoms, including short shake of the head and face (the first-stage seizure by Racine Score) showed yourself. However with repeated injection of between 12 to 15 infusions (average of 13 injections) rats with tonic-clonic seizures with loss of balance and even consciousness (the fifth stage of seizure based on Racine Score) (Table1). Kindling for approval, ten days after the last injection, the rats were injected again PTZ with the initial dose escalation and the seizure of the opinion that in the last injection, the animal was demonstrated, there was no difference.

**Table 1.** Injection of PTZ required to achieve each step of the Racine score (Mean ± SD)

| Seizure Score | Stage1 | Stage2 | Stage3 | Stage4 | Stage5 |
|---------------|--------|--------|--------|--------|--------|
| No. PTZ injection | 2.80 ± 0.78 | 5.20 ± 0.63 | 8.30 ± 0.67 | 11.00 ± 0.82 | 13.10 ± 1.20 |

**Western blotting**

In this study, the cerebellar PSA-NCAM expressions mean relative density was studied using Western blotting techniques. After normalization against β--actin, the PSA-NCAM mean relative density bands were estimated for all groups. As Figures 2 shows, on PD1, the cerebellar PSANCAM expressions in offspring were for Kindle+/Seizure+: 66.59±1.35, Kindle+/Seizure+: 68.66±1.15, Kindle+/Seizure−: 136.24±1.89, Kindle−/Seizure+: 137.15±1.24 as sham control and Normal groups: 139.15±2.85. Respectively, these values on PD14 were 33.60±1.14, 35.05±1.21, 66.97±1.08, 67.25±1.05 and 68.50±0.95. This shows that the cerebellar PSA-NCAM expression significantly decreased in neonates born from both groups mother (Kindle+/Seizure+ and Kindle+/Seizure−) that received PTZ-injection during pregnancy, compared with the sham control group (p=0.001 at PD1 and p=0.01 at PD14). However, the PSA-NCAM expression in the cerebellum of neonatal rats born from kindle and non- kindle mothers that never experienced seizures during pregnancy no showed any significant differences (p=0.48 at PD1 and p=0.25 at PD14).
Fig. 1. The rat pups cerebellar PSA-NCAM expression western blot analysis on PD1 (left) and PD14 (right). a: Normal group; b: Kindle-/Seizure the sham control group; c: Kindle+/Seizure- group; d: Kindle-/Seizure+ group; and e: Kindle+/Seizure+ group. The PSA-NCAM expression decreased significantly in Kindle+/Seizure+ and Kindle-/Seizure+ groups compared with the sham control and normal groups on both PD1 and PD14.

Fig. 2. The PSA-NCAM expression mean relative densities’ on PD1 and PD14 (Mean ±S.D).  
* P = 0.01   ** P = 0.001
Immunohistochemistry

Also evaluated effect of kindling and seizures during pregnancy on the cerebellar PSA-NCAM positive cells number per unit area in rat pup's on PD 1 and 14, in different groups. As Figures 3 shows the PSANCAM positive cells from the offspring cerebellum for Kindle+/Seizure+, Kindle+/Seizure−, Kindle−/Seizure−, Kindle−/Seizure+ as sham control and Normal groups on PD1 were 56.31±2.96, 59.18±2.35, 116.06±1.39, 118.06±1.44 and 119.18±2.42, respectively. After counting, these numbers on PD14 were 36.06±1.10, 37.31±1.26, 67.20±1.05, 67.68±1.60 and 68.56±1.30, respectively. This shows that cerebellar PSA-NCAM positive cell significantly decreased in neonates born from both groups mother (Kindle+/Seizure+ and Kindle+/Seizure−) that received PTZ-injection during pregnancy, compared with the sham control group (p=0.001 at PD1 and p=0.01 at PD14). However, cerebellar PSANCAM positive cells in neonates born from both groups mother Kindle+/Seizure− and sham control no showed any significant differences both on PD1 and PD14,(p=0.55 at PD1 and p=0.35 at PD14).

![Fig.3](image)

Fig.3. The cerebellar PSA-NCAM positive cells number per unit area in rat pups on PD 1 and 14 (Mean ± S.D).  * P = 0.01  ** P = 0.001
Fig.4. Coronal sections of the rat pups cerebellum: a) Photomicrographs of normal cerebellum with 10x, b) 40x, c) PSA-NCAM positive cell 100x, Scale bars) a:500 µm, b:100 µm and c:50 µm
Fig. 5. Photomicrographs of cerebellar PSA-NCAM Positive Cell of the rat pups on PD1 with 40x. a) Normal, b) Kindle⁺/Seizure⁻, c) Kindle⁻/Seizure⁺, d) Kindle⁻/Seizure⁺, and e) Kindle⁺/Seizure⁺. Scale bars: 100 µm
Fig. 6. Photomicrographs of cerebellar PSA-NCAM Positive Cell (Arrows) of the rat pups on PD14 with 40 x. a) Normal, b) Kindle+/Seizure−, c) Kindle+/Seizure+, d) Kindle−/Seizure+ and e) Kindle+/Seizure+. Scale bars: 100 µm
9. DISCUSSION

It has been proved that the highest cortical neurogenesis development during early prenatal takes place. This stage at 16 weeks of gestation in humans and in rats is 15 days after fertilization. Hippocampus and cerebellum, the structures of the nervous system that later evolve and complete formation in humans after 34 weeks of pregnancy and in rats during the first 2 weeks after birth. So if epileptic seizures occur during pregnancy, highly affect a neurogenesis process that impresses hippocampus and cerebellum development (13). Although damage in the cerebellum is not paralyzed, but disturbance in fine motor, balance and motor learning is created. Previously thought was that an epileptic seizure is a cerebrocortical phenomenon. However, yet the reports stating that the origin of seizures can cause cerebellum damage, including damage to the cerebellum ganglia. Previous studies have shown that for patients with epileptic seizures, serious damage to the white matter of the cerebellum into the relationship between the cerebellum and other areas of the cerebral cortex is faced with disorder (14). Since PSA-NCAM as a marker of neurons in the CNS is known evolution and migration, and considering the important role of the cerebellum in establishing communications with other parts to the brain such as the cortex, in this study the expression of this protein in the cerebellum infants born mothers with epilepsy were studied. The results showed that the seizures during a pregnancy lead to a significant increase in the infant mortality compared with sham control and normal groups. According to extensive studies of the impact of differences in terms of seizure on CNS nerve damage there. These differences may be due to differences in a model of epilepsy; frequency and severity of seizures, seizure induction time and the type of animal breed are studied (38). In this research, the effect of kindling and epileptic seizures during pregnancy on cerebellar PSA-NCAM expression in newborn's rat on PD 1 and 14 were evaluated. Western blot results show that PTZ-induced seizures significantly decrease cerebellar PSA-NCAM expression during gestational period in the neonatal rats born to both kindle and non-kindled seizure mothers compared with the sham control and normal groups. As well as, the effect of seizures during pregnancy on the cerebellar PSA-NCAM positive cells number per unit area in the babies born to mothers with epilepsy were studied. The results from our study show that PTZ-induced during pregnancy reduces PSA-NCAM positive cell on PD1 of kindle+/seizure+ and kindle/seizure+ cerebellum compared with kindle/seizure+ as sham control and normal groups. We observe similar results in PD14. According to Western blotting and immunohistochemistry results, the PSA-NCAM expression and cerebellar PSA-NCAM positive cell number in the babies born to
kindled mothers without experienced seizures during pregnancy compared with sham control and normal groups was not significant difference (p>0.05); It can be concluded that kindling not affect to PSA-NCAM expression and effective factor to PSA-NCAM expression was PTZ-induced seizure during pregnancy.

10. CONCLUSION
The results from this study demonstrate that a maternal epileptic seizure during pregnancy at non kindle and kindled mothers strongly reduces PSA-NCAM expression in the neonatal rat’s cerebellum. The decreased PSA-NCAM expression caused by seizures during pregnancy persists for 14 days after birth as an important period in rodent's brain and especially the cerebellum development. As regards, the PSA-NCAM expression is essential for motor learning; it is probably that a relationship between maternal seizures, reduction PSA-NCAM expression, and cerebellar structure and function in the offspring is there. However, more studies to determine epileptic seizure during pregnancy to describe the possible relationship between epilepsy and congenital malformations and mental retardation are necessary.

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