Seizures during pregnancy modify the development of hippocampal interneurons of the offspring

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A B S T R A C T

We investigated the effect of epileptic seizures during pregnancy on hippocampal expression of calcium-binding proteins in the offspring. Female Wistar rats were submitted to the pilocarpine model and mated during the chronic period. Seizure frequency was monitored over the entire pregnancy. Pups were perfused at postnatal days 6 and 13, and the brains processed for Nissl staining and immunohistochemistry for NeuN, calbindin, calretinin, and parvalbumin. Number of stained cells in the hippocampus was estimated through stereological methods. Our results showed a decrease in epileptic seizure frequency during pregnancy. No differences were observed in NeuN-positive, CR-positive cells, and Nissl-stained hippocampal neurons between the groups. However, there was a significant decrease in calbindin-positive cells (P = 0.005) and a significant increase in parvalbumin-positive cells (P = 0.02) in the experimental group when compared with the control group. These results suggest that seizures during pregnancy affect the development of specific hippocampal interneurons of the offspring.

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1. Introduction

It is estimated that one in 200 (0.5%) pregnant women has epilepsy [1]. Pregnant women with epilepsy are at increased risk for a variety of maternal complications, as are the fetus and particularly the fetal brain [2]. Pregnancy can change the course of epilepsy, altering seizure frequency, for example. Furthermore, epilepsy and antiepileptic drugs (AEDs) may have a significant impact on the course of pregnancy and the fetus, particularly the developing fetal brain [1]. Currently it is known that the relationships among maternal epilepsy, AED metabolism, teratogenicity, and perinatal effects must be considered simultaneously [3].

Children of mothers with epilepsy have lower perinatal vitality rates (APGAR scores) and higher perinatal mortality rates than the general population [4]. Moreover, children of mothers with epilepsy have lower birth weight compared with controls [5–12]. Different authors have observed a higher incidence of intrauterine growth retardation in children of both mothers with epilepsy treated with AEDs and those not treated, suggesting a possible direct effect of maternal epilepsy on the fetus [10, 13]. Other studies have also reported low birth weight and smaller head circumference in children of mothers with epilepsy, particularly those on polytherapy and also depending on ethnic, genetic, and environmental factors [4, 11]. Thus, there is a relationship between maternal epilepsy and the development of the fetus; however, it has not been established whether the epilepsy itself causes changes in the fetus or the AEDs are responsible for such changes.

Generalized seizures can be extremely deleterious to the mother and fetus as a result of the falls and anoxia to which both are exposed [14]. Tonic–clonic generalized seizures may be particularly harmful to the fetus, causing hypoxic injury of the central nervous system and other organs. Moreover, these seizures lead to elevation of maternal blood pressure and electrolytic changes, and may also result in spontaneous abortion or intrauterine death [15]. However, it is not known if seizures without fetal hypoxia cause damage [2]. Hallak et al. [16] found that seizures in pregnant rats lead to changes in hippocampal neurons, although the authors did not mention the type and duration of seizures and the occurrence of hypoxia. It is unclear whether other types of seizures, such as complex partial seizures and absence seizures, have similar negative consequences [2].

Most clinical studies investigating the relationship between epilepsy and pregnancy inevitably stumble on the interference by
antiepileptic therapy. However, the use of experimental models allows us to control variables such as epilepsy type, seizure frequency, age at pregnancy, inclusion or not of medication, among others. Thus, using the kainic acid model, our group found that seizure frequency during pregnancy was related to serum levels of estrogen and progesterone [17]. In addition, we observed a decline in seizure frequency during the initial two-thirds of the gestation and lactation periods, with frequency returning to the pregestation level after weaning [17]. Corroborating these data, using the pilocarpine model, we also observed that seizure frequency decreased significantly throughout pregnancy and in the first two-thirds of the lactation period [18].

The effect of maternal seizures on the developing brain of offspring, particularly on cortical structures, is poorly understood. In mammals, formation of the cerebral cortex is a temporally and spatially organized process, characterized by the formation of successive waves of proliferation, migration, differentiation, and cell death [19]. Each of these stages requires the balanced participation of various actors such as trophic factors, constituents of the cytoskeleton, signaling and cell adhesion molecules, and gene transcription factors. Failure in any one of these elements can potentially cause a disturbance of cortical development, resulting in more or less focal lesions, depending on the extent and type of defect involved [20, 21].

In the adult brain, immunohistochemistry for parvalbumin (PV), calbindin (CB), and calretinin (CR) reveals three distinct subpopulations of cortical neurons. PV, CB, and CR are calcium-binding proteins (CaBPs), the function of which in the nervous system is not fully known. However, these proteins are important because they can be used as markers of the main types of inhibitory interneurons (GABAergic) of the cerebral cortex [22]. Moreover, these neurons are involved in the equilibrium and control of brain excitability. Previous studies have shown that seizures during adulthood and postnatal development also alter the expression of these proteins and the organization of the interneurons that express them [23, 24].

In this article, we reported the effect of maternal seizures on brain development of the offspring, particularly the hippocampal formation, using the experimental model of pilocarpine-induced epilepsy in rats. Changes in inhibitory interneurons after birth were studied using the CaBPs PV, CB, and CR as markers.

2. Methods

The experimental protocols used in this study were approved by the Research Ethics Committee of the Federal University of São Paulo (No. 1151/03). All efforts were made to minimize animal suffering according to international ethical guidelines [25].

2.1. Animals

Wistar rats, males and females, were housed in groups of five under controlled environmental conditions (light–dark cycle of 12 hours, lights on between 7 AM and 7 PM, and constant temperature of 20–22 °C), with free access to water and food.

2.2. Vaginal cytology

Female virgin Wistar rats, weighing 200–250 g, were controlled for the pattern of estrous cycle by checking the characteristics of vaginal cytology. Vaginal contents were obtained using a nontraumatic dropper containing saline. The solution obtained from the vaginal conduit was placed on a slide and observed with a microscope. This procedure was always performed between 8 and 10 AM. Only rats with a regular estrous cycle, with respect to onset of estrus stage at intervals of 3 to 4 days, were included in the study.

2.3. Study groups

Before mating, selected females were divided into two groups each containing six rats. The first group consisted of females submitted to the pilocarpine model (see below), and the second group comprised control females. During the study we had to create a third group, as females with epilepsy exhibited cannibalism with their own offspring, and we had to exchange the litters (cross-fostering) between females with and without epilepsy soon after birth.

2.4. Pilocarpine-induced epilepsy model

The pilocarpine model was used as described in previous studies [18, 26, 27]. Briefly, selected females were pretreated with a subcutaneous injection of methylscopolamine (1 mg/kg, subcutaneously, Sigma) 30 minutes before administration of pilocarpine, to minimize peripheral cholinergic effects. After this interval, experimental animals received an injection of pilocarpine (350 mg/kg, intraperitoneally, Merck). Control animals received the same treatment, except pilocarpine was replaced with a saline solution (NaCl 0.9%). The status epilepticus (SE) induced with pilocarpine was not blocked. After SE, the animals received special care including hydration and a fractionated diet. Surviving animals were monitored continuously to detect spontaneous seizures using a video recording system. After the first spontaneous seizure, seizure frequency was controlled for 1 week and then the rats were placed for mating.

2.5. Mating and pregnancy

The females were placed in cages with males during the period of estrus. Vaginal contents were analyzed daily at 8 AM and 6 PM. The presence of sperm was used as a marker for the onset of pregnancy (day 0) and separation of the couple. During pregnancy, females with epilepsy were housed in separate cages (transparent acrylic cylinder) and video-monitored 24 hours/day to determine seizure frequency. During this period, the weight of females was recorded weekly to rule out maternal malnutrition.

2.6. Immediate postpartum period and cross-fostering

Immediately after birth, the litters were subjected to cross-fostering, the exchange of the offspring of rats with and without epilepsy. This process was carried out with extreme caution so that the pups could be adopted by the rats with the least stress possible. The offspring of a group were removed from the box with large anatomical tweezers and placed one by one with a female of another group recently separated from her own offspring. Cross-fostering was similarly carried out with the control rats to reduce the influence of adoption-related stress on the growth and development of the offspring. To prevent rejection, these animals were handled only after 48 hours of postnatal life (postnatal day 2 [P2]). All litters were reduced to eight pups per female to ensure adequate nutrition of the offspring. In the present study, only male pups were used.

2.7. Histology and immunohistochemistry

Brains were studied at P6 and P13. Pups were anesthetized with 0.1 mL of chloral hydrate 4% and subjected to transcardiac perfusion with a solution of paraformaldehyde 1% (pH 7.4, 15 mL/rat, infusion rate 15 mL/minute) followed by a solution of paraformaldehyde 4% (pH 7.4, 150 mL/rat, infusion rate 15 mL/minute). After perfusion, the brain was carefully removed from the skull, postfixed in paraformaldehyde 4% for 48 hours, and immersed in a solution of sucrose 30% for cryoprotection for 48 hours. Fifty-micrometer-thick coronal slices were obtained using a cryostat (HM 505E Micromeria, Zeiss) and stored in 0.1 M phosphate buffer (pH 7.4). The slices were collected throughout the hippocampus...
and stored in 0.1 M phosphate buffer. One of ten slices (1:10) was mounted on gelatin-coated slides for Nissl staining with cresyl violet. Adjacent slices were selected for immunohistochemistry with the antibodies Neun (1:1000-NerveChemical-1:10 sections), CR (1:2000-Swant Swiss-1:10 sections), CB (1:2000-Swant Swiss-1:10 sections), and PV (1:5000-Swant Swiss-1:5 sections).

The immunohistochemistry was performed in the same way for all primary antibodies in P6 (Neun, CB, CR) and P13 (PV) pups. Briefly, free-floating slices were treated with hydrogen peroxide 1% for 10 minutes, washed with phosphate-buffered saline (PBS, pH 7.4) several times, and then treated with Triton X-100 0.4% for 30 minutes. Slices were washed with PBS, pre-incubated with albumin 10% for 2 hours, and incubated with primary antibody overnight at 4°C. The next day, the slices were washed and then incubated at room temperature with appropriate secondary antibodies (1:200, biotinylated immunoglobulin G, Calbiochem) for 2 hours. Sections were well washed and incubated in avidin–biotin–peroxidase complex (ABC Kit, Vector) for 90 minutes, washed again with Tris–HCl (pH 7.6), and finally developed with diaminobenzidine (DAB, 1 tablet/15 mL of Tris medium). Sections were dehydrated in ethanol, mounted on gelatin-coated slides for Nissl staining with cresyl violet.

### 2.8. Stereological cell counting

The number of hippocampal neurons was estimated using the stereological method “optical fractionator” [28]. Briefly, one of five (1:5) or one of ten (1:10) sections was selected, resulting in a section sampling fraction of 0.2 (ssf = 0.2) or 0.1 (ssf = 0.1). In each section, both hippocampi were identified according to a brain atlas [29]. Dissector counting probes were uniform and randomly distributed through the hippocampus, including all subfields of the cornu ammonis (CA1, CA2, CA3) and the hilus of the dentate gyrus. The granular cell layer was not included in the present investigation for technical reasons. Depending on the cellular population to be counted, the area (a) of the probe (dissector) varied from 625 to 10,000 μm², and the distance between counting frames was 250 or 500 μm (Table 1). Neuronal cell bodies (tops) were counted through the entire thickness of each section, resulting in a thickness sampling fraction of 1 (tsf = 1). The total neuronal cell number (N) for each region was estimated using the formula [28]

\[
N = \sum Q^{-1} \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{tsf}
\]

where \(Q\) is the number of neurons counted, tsf is the thickness sampling fraction, asf is the area sampling fraction, and ssf is the section sampling fraction. A pilot study showed that this sampling scheme produced acceptable coefficients of error (CEs) and variance (CVs) [28, 30]. Estimates for cresyl violet- and NeuN-stained cells were made at P6. Estimates for CaBP-stained cells were made at P6 (CB and CR) or P13 (PV).

### 2.9. Statistical analysis

The results were analyzed with the following tests, as appropriate: analysis of variance (ANOVA) followed by Tukey test, Student’s t test, and \(\chi^2\) test.

### 3. Results

#### 3.1. Monitoring of pregnancy

Data for the epilepsy model induced with pilocarpine in female rats were similar to previous data [18]. In rats with epilepsy, the mean weekly seizure frequency observed before pregnancy was 20.8 ± 5.5 (n = 11). This frequency was significantly reduced during the entire pregnancy, particularly during the first week (seizure frequency: 4.3 ± 1.0, n = 11, P < 0.01, Student’s t test). Seizure frequency was also lower during the second (8.3 ± 2.5, n = 11, P < 0.05, Student’s t test) and third (7.2 ± 2.2, n = 11; P < 0.05, Student’s t test) weeks. These data were previously reported by us [31] as the pups used in the present work were obtained from these mothers. It is interesting to note that the medium incidence of seizures throughout the pregnancy in 3 weeks (18.3 ± 4.5, n = 11) was similar to that observed in only 1 week before pregnancy (20.8 ± 5.5, n = 11). Seizure duration during pregnancy ranged from 2 to 38 seconds (18.3 ± 1.4 seconds).

Pregnancy lasted 21.3 ± 0.25 days in the control group and 21.3 ± 0.18 days in the experimental group (no difference between groups). There was no significant difference in weight gain during pregnancy between the control and experimental groups (data not shown). No abortions were observed in control and epileptic rats. The interval between status epilepticus and pregnancy was 3 to 6 months.

#### 3.2. Immediate postpartum period

The number of pups born in each litter in the control group (11.1 ± 0.7, n = 12) was similar to the number of pups in the experimental group (9.5 ± 0.6, n = 13, P = 0.0794, Student’s t test). The proportion of males was 52% in the control group and 50% in the experimental group (P = 1.0, Fischer). Dead pups were observed in 50% of the litters of experimental rats (5 of 10 litters, 1 pup/litter). In these cases, there were hematomas throughout the body. Dead pups were not observed in the control group. The metric parameters of pups at P2 differed between the experimental animals without cross-fostering (n = 20), the experimental animals with cross-fostering (n = 20), and the control animals (n = 20): weights were 5.32 ± 0.17a, 6.12 ± 0.17b, and 6.42 ± 0.08; lengths in millimeters were 47.15 ± 0.55a, 50.75 ± 0.46b, and 53.35 ± 0.24; anteroposterior distances in millimeters were 14.3 ± 0.17a, 17.06 ± 0.11b, and 17.15 ± 0.13; and laterolateral distances in millimeters were 8.95 ± 0.15b, 8.93 ± 0.14a, and 9.75 ± 0.09, respectively. (Values are means ± SE. \(P < 0.001\): experimental without cross-fostering × control; \(P < 0.01\) and \(P < 0.001\): experimental with cross-fostering × control; \(P < 0.01\) and \(P < 0.001\): experimental without cross-fostering × experimental with cross-fostering.)

#### 3.3. Histology, immunohistochemistry, and cell counting

General brain morphology was macroscopically normal in the experimental and control groups. The internal organization and cytoarchitecture of different brain regions were preserved. Numbers of Nissl-stained neurons at P6 were similar in control and experimental pups. There was also no statistically significant difference in the number of NeuN-positive neurons between control and experimental rats.

Calretinin-positive cells in control pups at P6 were observed in all layers of CA1 and CA2, in the pyramidal layer of CA3, and throughout the dentate gyrus. This aspect was similar in control and experimental pups at P6 (Fig. 1). There was no statistically significant difference in number of CR-positive cells between control and experimental rats.

Calbindin-positive cells and neuropil in control pups at P6 were intensely stained in the granular cell layer and molecular layer of the dentate gyrus. CB immunoreactivity was diffuse in CA1 and CA2, more evident in stratum oriens. In the experimental group, CB immunoreactivity was decreased in all hippocampal regions, particularly the

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**Table 1**

<table>
<thead>
<tr>
<th>Marker</th>
<th>(a (\mu m^2))</th>
<th>(A (\mu m^2))</th>
<th>asf</th>
<th>ssf</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cresyl violet</td>
<td>25 ± 25</td>
<td>250 ± 250</td>
<td>1/100</td>
<td>1/100</td>
<td>0.03</td>
</tr>
<tr>
<td>Neun</td>
<td>25 ± 25</td>
<td>250 ± 250</td>
<td>1/100</td>
<td>1/100</td>
<td>0.04</td>
</tr>
<tr>
<td>Calretinin</td>
<td>50 ± 50</td>
<td>250 ± 250</td>
<td>1/6.25</td>
<td>1/100</td>
<td>0.05</td>
</tr>
<tr>
<td>Calbindin</td>
<td>100 ± 100</td>
<td>250 ± 250</td>
<td>1/6.25</td>
<td>1/100</td>
<td>0.01</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>100 ± 100</td>
<td>500 ± 500</td>
<td>1/5</td>
<td>1/5</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Note: \(A\) = area of the dissector, \(A =\) dissector interval, asf = area sampling fraction, ssf = section sampling fraction, CE = coefficient of error.*
dentate gyrus. Calbindin-positive blood vessels were also found in all hippocampal regions (Fig. 1). There was a significant decrease in CB-positive cells in experimental rats compared with the control group.

Parvalbumin-positive cells and neuropil in control rats at P13 were intensely stained in the pyramidal layer of CA1 to CA3, the granular cell layer, and the hilus of the dentate gyrus. PV-positive neuronal processes, mainly dendrites, were also observed throughout the hippocampus. In the experimental group, PV immunoreactivity was increased in all hippocampal regions (Fig. 1). The number of PV-positive cells in experimental rats was significantly higher than that in the control group.

4. Discussion

Several authors have studied the effect of maternal seizures on offspring in experimental models. Using the kindling model, Berzaghi [32] found that the offspring of rats with low seizure frequency during pregnancy had low weights on the 7th day of postnatal life (P7).
Mortality during the first 2 weeks of life was observed in 17% of litters. Pups of rats with frequent seizures during pregnancy had high weights from birth to the 21st postnatal day (P21). In this group, mortality was observed in 50% of litters, mainly within the first 48 hours of life [32]. In the same experimental model, the levels of biogenic amines and their metabolites in different brain structures were altered after birth [33]. In the pilocarpine model [18], number, weight, and proportions of males and females were similar in the offspring of rats with and without epilepsy. Stillbirths or birth defects were not observed, and general motor activity and vocalization were similar in both groups [18].

In the present study, we observed that general brain morphology and internal organization and cytoarchitecture were preserved in the offspring of rats with epilepsy. Specifically in the hippocampus, there was no significant neuronal loss, considering the structure as a whole. Interestingly, we observed a large difference between the number of Nissl-stained neurons and the number of NeuN-positive neurons. This difference could be explained by a decrease in the expression of NeuN or any technical factor. In fact, Unal-Cevik et al. [34] found that after mild cerebral ischemia, NeuN expression was significantly reduced in tissue subjected to immunohistochemistry, but remains unchanged when measured by Western blot. So far, there is no explanation for this effect.

Despite the apparent preservation of brain structure and absence of cell loss, the offspring of epileptic rats had a change in specific neuronal subpopulations. The number of CB-positive interneurons was lower and the number of PV-positive interneurons was higher in experimental rats, compared with the control group. These differences suggest abnormal development or differentiation of these neurons. Indeed, changes in the development of inhibitory interneurons have been observed in different studies. Mallard et al. studied sheep subjected to hypoxic insults during pregnancy by clamping the umbilical cord and observed a marked reduction in calbindin and parvalbumin expression in the offspring [35]. Similarly, Gerstein et al. observed a reduction in PV-positive neurons in the hippocampus of the offspring of rats subjected to hypoxia during pregnancy [36]. In the same study, the density of PV- and CB-positive neurons was also reduced in deep layers of motor cortex [36]. Studies in rats subjected to neonatal hypoxia showed a reduction in GABAergic neurons, but no change in the number of CB-positive neurons in the hippocampus [37, 38] and striatum [39]. These results point to the greater susceptibility of interneurons that express CaBPs (inhibitory neurons) to hypoxic–ischemic insults occurring during early brain development.

The change in expression of CaBPs that we observed in the hippocampus of the offspring of epileptic rats could be associated with hypoxic–ischemic insults suffered by those pups during maternal seizures. It is interesting to note that the increase in maternal seizures that occurs in the second week of pregnancy, as compared with the first week, is coincident with the beginning of CB expression [40]. Therefore, maternal seizures may have deleterious effects on the development of neurons that express CB. Another important factor, the stress reaction resulting from maternal seizures, could be involved in the brain changes we observed in the offspring, as the increase in plasma levels of glucocorticoids can alter brain development [41]. Moreover, gene and protein expression of CaBP in the brain may be regulated by glucocorticoids. For example, adrenalectomized rats manifest a drastic decrease in expression of CB (mRNA and protein) in the hippocampus, and this change is completely reversed by the administration of exogenous glucocorticoids [42]. On the other hand, maternal stress induced during the last week of pregnancy, with increased blood levels of glucocorticoids, induces a significant reduction in CB expression in the hypothalamus of the offspring [43]. Our results corroborate these studies and suggest that the effect of glucocorticoids on the brain varies with age.

In this study, in addition to a reduced number of CB-positive neurons, we observed ectopic expression of this protein in blood vessels in the offspring of epileptic rats. This type of ectopic expression was previously observed in adult rabbits subjected to hypoxia [44]. The authors observed the expression of CB in the basal lamina of microvessels of the anterior horn of the spinal cord about 30 minutes after ischemia, with an increase in immunoreactivity up to 3 hours after the insult. The authors suggest that the ectopic expression of calbindin is possibly related to calcium buffering by endothelial cells after ischemic insult [44]. Data from our group showed placental lesions suggestive of hypoxia–ischemia in epileptic female rats after delivery [31]. Thus, we assume that the CB expression observed in brain vessels of experimental pups could be an adaptive process of neuroprotection.

Contrasting with CB expression, PV immunoreactivity in hippocampal neurons is late, and can be observed only in the second week of postnatal life [38, 45]. Moreover, several authors have observed a lower susceptibility of PV-positive neurons to cell death after hypoxic insults, during either the fetal period [35], neonatal life [38], or adulthood [46]. Thus, the increased number of PV-positive neurons observed in the offspring of epileptic rats could be a result of adaptive changes in response to intrauterine hypoxic–ischemic insults and stress experienced during the fetal and neonatal periods. This increase in PV expression could be a neuroprotective mechanism.

Stress hormones are potent regulators of cell development and differentiation. For example, synthetic steroids are widely used in women at risk for premature birth, to accelerate the development of the lungs of the fetus [47]. In the brain, prenatal treatment with exogenous corticosteroids has effects on hippocampal cell proliferation, neurotransmitter turnover, and receptor expression [48, 49]. Therefore, another explanation of the changes in CaBP expression in this study may be an anomalous anticipation of brain development induced by stress and hypoxia caused by maternal seizures. In normal brain development, the early expression of PV spatially and temporally coincides with the reduction in expression of CB observed between the second and third postnatal weeks [50]. During this period, a subpopulation of cortical interneurons stops expressing CB and start to express PV. Thus, the reduction in CB-positive neurons and the increase in PV-positive neurons observed in offspring of epileptic rats could result from anomalous anticipation of brain maturation induced by maternal seizures. This hypothesis is supported by the work of Silva et al. [24], who observed a similar phenomenon in CB- and PV-positive cortical neurons after the induction of seizures in rats during early postnatal life (P7, P8, and P9). As there was no change in calretinin expression it is possible that these subsets of interneurons are not affected in this experimental model.

Seizures during pregnancy affect development of the brain in the offspring. Although we have not observed gross malformations or neuronal loss, we observed a change in the development of hippocampal interneurons that express CaBPs. This result is important because it may reflect an imbalance in the mechanisms of excitation and inhibition, but further studies are needed to determine the effect of this imbalance in brain excitability, as well as cognitive processes, learning, and memory.

5. Ethical approval

We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

6. Conflict of interest statement

None of the authors has any conflict of interest to disclose.

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