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Time course of the effect of *status epilepticus* induced in the developing rat on γ-amino butyric acid and glutamate cerebellar concentration☆

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Abstract
Introduction: *Status epilepticus* (SE) is an epileptic condition that can cause cerebellar atrophy and loss of Purkinje cells in both humans and research animals. Cerebellum is a region rich in γ-amino butyric acid (GABA) and glutamate, and some studies have shown that their concentrations may be altered after convulsions. However, there are no studies showing the effect of seizures on different cerebellar regions in developing rats. Time course of the effect of status epilepticus induced in the developing rat on γ-amino butyric acid and glutamate cerebellar concentration.
Methods: SE was induced using the lithium-pilocarpine model; control rats were injected with saline solution. At 6 hours, 24 hours, and 1 month after SE o saline injection, rats were anaesthetised with pentobarbital and decapitated, and cerebella were extracted. The vermis and hemispheres were dissected and homogenised in 0.1 M perchloric acid containing 4 mM sodium bisulfite. Homogenates were centrifuged and supernatant was used to quantify GABA, and glutamate tissue concentrations by HPLC coupled with fluorometric detection.
Results: SE did not alter GABA and glutamate tissue concentration in the cerebellar vermis and hemispheres.
Conclusion: The developing rat cerebellum is resistant to both short- and long-term neurochemical changes induced by SE.

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PALABRAS CLAVE
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Cerebelo;
Ácido γ-aminobutírico;
Glutamato

Evaluación temporal del efecto del *status epilepticus* inducido en la rata en desarrollo en la concentración cerebelar de ácido γ-aminobutírico y glutamato

Resumen
Introducción: El *status epilepticus* (SE) es un tipo de actividad epiléptica que causa atrofia cerebelar y pérdida de células de Purkinje en humanos y en animales de experimentación. El cerebelo es una región con alto contenido de ácido γ-aminobutírico (GABA) y glutamato, y algunos estudios refieren cambios en su concentración después de las convulsiones. Sin embargo, hasta la fecha no existen estudios que hayan analizado su efecto en diferentes regiones cerebelares en ratas en desarrollo. El objetivo del presente estudio fue realizar un curso temporal del efecto del SE inducido en ratas Wistar de 14 días de edad (P14) sobre el contenido tisular de GABA y glutamato en el vermis y los hemisferios cerebelares.
Métodos: El SE se indujo con el modelo de litio-pilocarpina; las ratas control se inyectaron con salina. Seis h, 24 h o 30 días después del inicio del SE o de la aplicación de solución salina, las ratas se anestesiaron y decapitaron, se extrajo su cerebelo y se separaron el vermis y los hemisferios. Las ratas de ambos grupos se anestesiaron y decapitaron, se extrajo su cerebelo y se separaron el vermis y los hemisferios. Ambas regiones se homogeneizaron (ácido perclórico 0,1 M conteniendo metabisulfito de sodio 4 mM) y centrifugaron, y el sobrenadante se empleó para cuantificar la concentración tisular de GABA y glutamato por cromatografía de líquidos de alta resolución acoplada a un detector fluorométrico.
Resultados: El SE no modificó la concentración de GABA y glutamato a los diferentes tiempos de análisis ni en el vermis ni en los hemisferios cerebelares.
Conclusiones: El cerebelo en desarrollo es resistente a los cambios neuroquímicos a corto y largo plazo producidos por el SE.

Introduction

The International League Against Epilepsy recently defined status epilepticus (SE) as a condition resulting either from the failure of the mechanisms responsible for seizure termination or from the initiation of mechanisms that lead to abnormally prolonged seizures. The long-term consequences of SE depend on seizure type and duration and include neuronal death, neuronal damage, and alteration of neural networks.[1] SE is considered the most extreme form of epilepsy.[2] Its incidence varies according to a number of factors, including age, ethnicity, genetics, and socioeconomic status.[3] Five epidemiological studies conducted in specific populations in Europe and the United States report a higher incidence of convulsive SE in children younger than one or 5 years.[3] This suggests that the paediatric population is particularly vulnerable to this type of epilepsy and its consequences.

Cerebellar development begins during the embryonic phase and finishes after birth.[4,5] This structure is involved in multiple functions, including balance, movement and postural control, and motor learning[5,6]; it has also been associated with sexual and addictive behaviour.[7–10] Unlike such other central nervous system diseases as ataxia and autism,[11,12] epilepsy has not traditionally been associated with cerebellar function. However, some studies have shown that the modulation of cerebellar function may have antiepileptic effects.[13–16] Furthermore, patients with epilepsy or a history of SE display cerebellar atrophy[17–20] and Purkinje cell loss[21–23]; this has also been observed in experimental models of epilepsy.[24–26] The cerebellum is rich in amino acids, mainly γ-amino butyric acid (GABA) and glutamate: Purkinje and granule cells (the most abundant types of cells in the brain) use these amino acids as inhibitory and excitatory neurotransmitters, respectively, to integrate information transmitted to and from the brain.[27]

Little is known about the consequences of epileptic activity on the neurochemistry of the cerebellum, especially in the developing brain. Induction of febrile seizures in 10-day-old rats results in decreased GABA, taurine, and alanine concentrations and increased levels of aspartic acid in the whole cerebellum 24 hours after seizures.[28] Induction of SE in 14-day-old rats has been found not to change tissue GABA or glutamate concentration in the cerebellum 24 hours after seizures.[29] These findings underscore the importance of studying whether the changes in the concentration of these neurotransmitters are observable immediately after SE, or whether they occur over a longer period. This study aimed to describe the time course of the effects of lithium-pilocarpine-induced SE on tissue concentrations of GABA and glutamate in the vermis and hemispheres of the developing rat cerebellum.

Material and methods

Experimental subjects

We studied 36 male and female Wistar rats of 14 days postnatal age (P14) at the time that seizures were induced (body weight 25-30 g); the rats were raised in the vivarium of the Brain Research Centre at Universidad Veracruzana. The day of birth was considered postnatal day 0 (P0). Newborn rats were kept with their mothers in transparent acrylic cages measuring $15 \times 24 \times 37$ cm until the end of the experiment or until weaning on postnatal day 21. Adult rats were all male and were housed in collective acrylic boxes measuring $20 \times 30 \times 50$ cm. All rats were housed at ambient temperature and humidity levels with 12:12 light/darkness cycles starting at 8:00 am, and had free access to food and water. All experiments observed domestic and international standards and complied with the official Mexican guidelines on the use and care of experimental animals (NOM-062-ZOO-1999) and the National Research Council guide for the care and use of laboratory animals (2011 version).

Experimental groups

Rats were divided into 6 groups ($n = 6$ per group). They were euthanised 6 hours, 24 hours, or 30 days after induction of SE; control rats were manipulated and euthanised at the same time points as the experimental rats.

Induction of status epilepticus with lithium-pilocarpine

Rats were injected with lithium chloride (3 mEq/kg, intraperitoneal administration) on day P13; 20 hours later, on day P14, SE was induced with pilocarpine chlorhydrate (100 mg/kg, subcutaneous administration). We used the scale proposed by Haas et al.[30] to monitor behavioural manifestations of SE; only animals displaying this behaviour were included in the study. Control rats were injected with lithium chloride and saline solution. All rats were rehydrated with glucose solution (5%) 7 hours after the experiment started (1 mL, subcutaneous administration) and were immediately returned to their mothers. We chose this time point because seizures had stopped after 7 hours in the rats with SE. Experimental and control rats were separated from their mothers for similar periods of time.

Tissue processing

Rats were anaesthetised with pentobarbital sodium (60 mg/kg, intraperitoneal administration). Following decapitation, the cerebellum was dissected on ice (approximately 4 °C), and the vermis was separated from the hemispheres (both hemispheres were processed together). Brain tissue was then mechanically homogenised by adding 30 μL perchloric acid 0.2 N ($HClO_4$, Baker) and sodium metabisulfite (S1516, Baker) for every 10 mg of tissue. Tissue homogenates were centrifuged at 10 000 rpm for 20 minutes at 4 °C; the supernatant was collected and filtered through a 0.45-μm pore-size HV filter (Millipore). The filtrate and sediment were stored at −70 °C until amino acid and protein quantifications were performed.

Determination of tissue amino acid concentration using high-performance liquid chromatography

GABA and glutamate concentrations in the vermis and hemispheres of the cerebellum were quantified according to the procedure described by Luna-Munguía et al.[31] High-performance liquid chromatography was performed using a Waters 474 scanning fluorescence detector, a Nova-Pak C18 pre-column (4 μm-60 A [3.9 × 20]), and a reversed-phase Nova-Pak C18 column (4 μm-60 A [3.9-150]). The fluorescence detector operated at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. For amino acid quantification, we prepared a supernatant dilution (1:250-1:450), mixed 20 μL of the dilution with 6 μL of the derivatisation reagent, and injected the mixture manually (25 μL) into the chromatograph 2 minutes after the reaction started. The derivatisation reagent was prepared by mixing 4.7 mg o-phthalaldehyde (OPA), 94 μL methanol, 0.874 mL potassium tetraborate 0.4 M, and 8.8 μL 2-mercaptoethanol 0.116 M. Chromatography was performed with a binary gradient system. Mobile phase A was a buffer solution of sodium acetate 40 mM in 10% methanol (pH 6.7). Mobile phase B was a buffer solution of sodium acetate 8 mM in 80% methanol (pH 5.7). The elution profile was as follows: 77% A and 23% B at 0 min; 55% A and 45% B at 1 min; 30% A and 70% B at 6.5 min; 3% A and 97% B at 11-13 min; 77% A and 23% B at 16 min (flow rate: 0.5 mL/min). GABA and glutamate concentrations were determined with linear regression analysis using the Millennium package (Waters®) based on an external standard calibration curve (100, 300, and 500 ng/mL) of GABA and glutamate. Amino acid concentration was expressed in pg/mg protein.

Protein quantification using the Bradford protein assay

Proteins were quantified with the Bradford protein assay (Quick Start Bradford protein assay kit 1x dye reagent, Bio-Rad) using the sediment of the homogenised cerebellum. To this end, we prepared a standard curve with bovine serum albumin (Sigma). Absorbance was determined using a SpectraMax 190 microplate reader (Molecular Devices) at a wavelength of 595 nm.

Statistical analysis

The results were analysed with two-way ANOVA for independent samples; the independent variables were treatment (SE vs control) and assessment time point (6 hours, 24 hours, and 30 days), and the dependent variables were GABA and glutamate concentrations. The Tukey test for multiple comparisons was used to identify differences between factors. Statistical analysis and graphing were performed with GraphPad Prism 5.0. Results are presented as means ± standard error of the mean (SEM); statistical significance was set at $P < .05$.

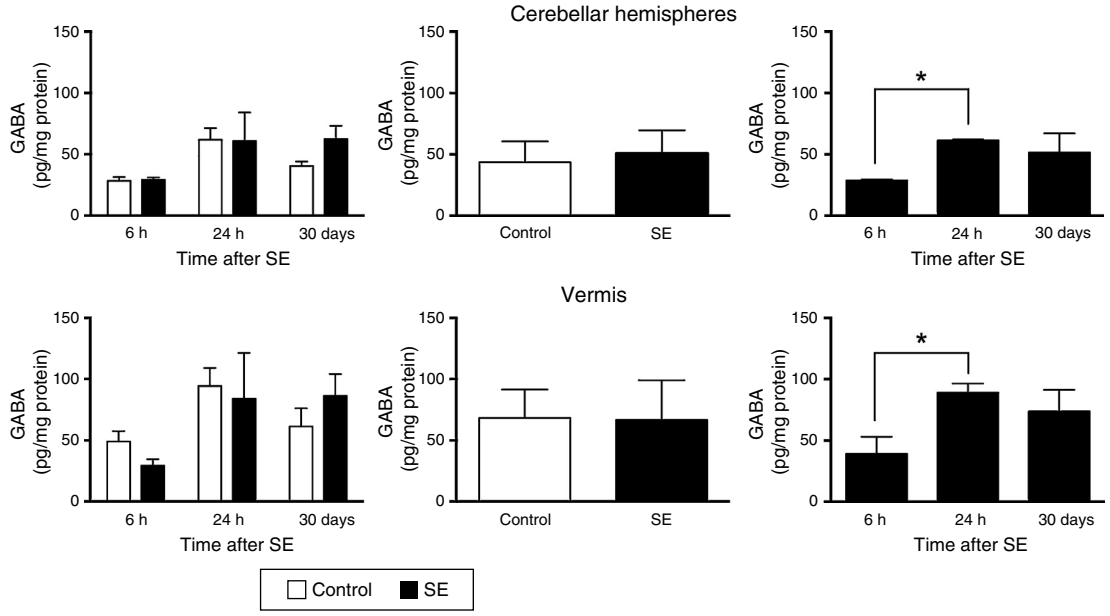

Figure 1 GABA (γ-amino butyric acid) concentration in the cerebellar hemispheres and vermis after status epilepticus (SE). The graphs on the left show the time course of GABA concentration after SE. The graphs in the centre show the effect of treatment (induced SE or sham manipulation) on GABA concentration regardless of time. The graphs on the right show GABA concentration at different time points, regardless of treatment. Bars indicate mean (±SEM) GABA concentration ($n = 6$ rats per group). *$P < .05$.

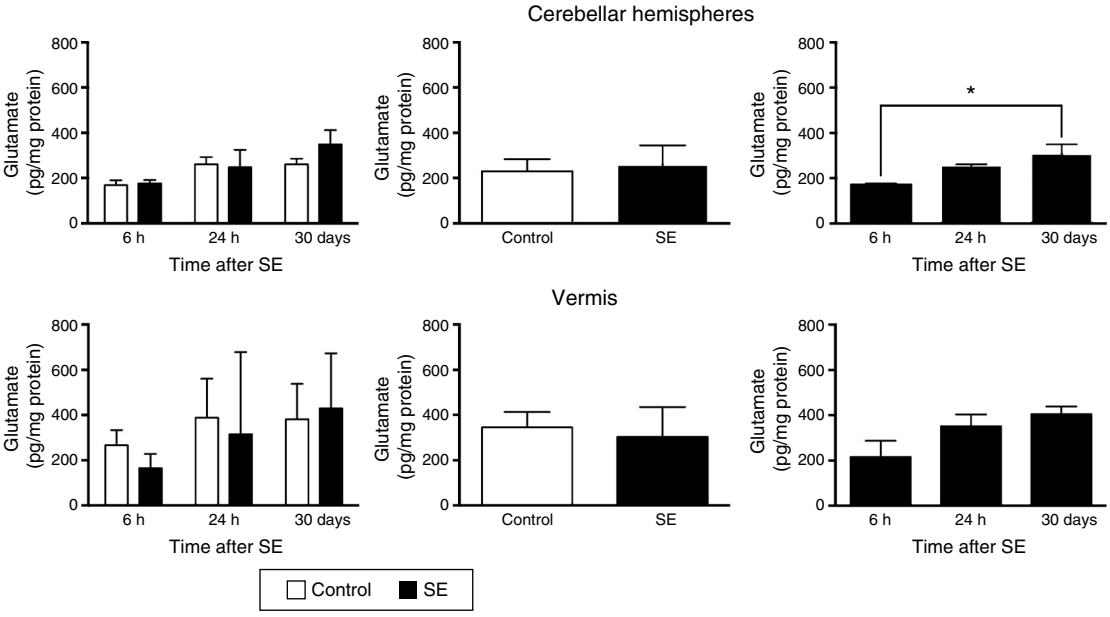

Figure 2 Glutamate concentration in the cerebellar hemispheres and vermis after status epilepticus (SE). The graphs on the left show the time course of glutamate concentration after SE. The graphs in the centre show the effect of treatment (induced SE or sham manipulation) on glutamate concentration regardless of time. The graphs on the right show glutamate concentration at different time points, regardless of treatment. Bars indicate mean (±SEM) glutamate concentration ($n = 6$ rats per group). *$P < .05$.

Results

Status epilepticus lasted 5 to 6 hours. Maximum seizure severity in our sample was stage 5; rats displayed generalised seizures characterised by bilateral forelimb clonus and rearing, with or without loss of postural tone.

The two-way ANOVA revealed differences in tissue GABA concentrations in the cerebellar hemispheres; these differences were dependent on the assessment time point ($F_{(2,30)} = 4.47$; $P = .0199$) but not on treatment ($F_{(1,30)} = 0.645$; $P = .4282$) or the interaction between treatment and assessment time point ($F_{(2,30)} = 0.651$; $P = .5299$). A similar effect

was observed in the cerebellar vermis: statistical analysis revealed differences in tissue GABA concentration, which were dependent on the time of assessment ($F_{(2,30)} = 3.5$; $P = .0430$) but not on treatment ($F_{(1,30)} = 0.010$; $P = .9190$) or the interaction between treatment and time of assessment ($F_{(2,30)} = 0.749$; $P = .4813$). The post hoc test revealed higher GABA concentrations in the cerebellar hemispheres and vermis ($P < .05$) at 24 hours than at 6 hours (Fig. 1).

Similarly, the differences in tissue glutamate concentration in the cerebellar hemispheres were found to depend on the time of assessment ($F_{(2,30)} = 4.37$; $P = .0216$) but not on treatment ($F_{(1,30)} = 0.556$; $P = .4615$) or the interaction between treatment and time of assessment ($F_{(2,30)} = 0.710$; $P = .4995$). According to post hoc test results, glutamate concentration in the hemispheres is lower at 6 hours than at 30 days ($P < .05$). Glutamate concentration in the cerebellar vermis did not change in relation to time ($F_{(2,30)} = 2.67$; $P = .0855$), treatment ($F_{(1,30)} = 371$; $P = .5473$), or the interaction between these 2 factors ($F_{(2,30)} = 0.44$; $P = .6481$) (Fig. 2).

Discussion

Our study shows that SE induced in 14-day-old rats does not result in either acute or long-term changes in GABA or glutamate concentrations in either the vermis or the hemispheres of the cerebellum.

The causes and consequences of SE have been studied with experimental models mimicking this type of epileptic activity. We used the lithium-pilocarpine model to study SE during rat development as this technique causes motor symptoms as well as neuronal damage in different brain regions.[32–35] Our results show that the neurochemistry of GABA and glutamate in the cerebellum of developing rats is not significantly affected by SE either immediately after seizure, 24 hours after the episode, or in the long term. Few studies have evaluated the effect of SE on the extracellular concentration of cerebellar amino acids. In a previous study, SE induced in 14-day-old rats did not change GABA or glutamate concentrations in the cerebellar vermis or hemispheres, but did increase alanine, taurine, and glutamine levels in the cerebellar hemispheres 24 hours after the episode. This effect was accompanied by a lack of change in radioligand binding to the $GABA_A$ receptor in the cerebellar vermis.[29] The induction of febrile seizures in 10-day-old rats is known to increase aspartate levels in the cerebellum, whereas the concentration of such other amino acids as GABA and glutamate decreases 1 day after the seizure.[28] The fact that SE does not affect GABA or glutamate concentrations suggests that the developing cerebellum resists changes induced by seizures, even seizures lasting several hours. This may be accompanied by changes typically occurring during the development of the rat brain or associated with brain plasticity.[36] In fact, the anatomical patterns and the intensity of SE-induced neural damage in rats are known to depend on the age at which SE is induced. Spontaneous epileptic seizures due to SE are less frequent in rats in which SE is induced at the age of 2 weeks than at ages of 3 or 4 weeks.[32]

According to our results, GABA concentration in the vermis and cerebellar hemispheres was higher when evaluated 24 hours after seizure (or after manipulation with saline solution, in the case of control rats), when rats were 14 days old, than 6 hours after sample collection, when they were 13 days old. Glutamate concentration was higher 30 days after SE or sham manipulation than at 6 hours. This suggests that changes may be due to rat cerebellar maturation itself; marked cell proliferation and neurogenesis have been observed to extend until approximately postnatal day 25.[37] Furthermore, GABAergic synapses are known to form during the second and third weeks of life.[38]

In conclusion, this study shows that induced SE causes neither short- nor long-term changes to GABA or glutamate concentration in the vermis and hemispheres of the cerebellum of 14-day-old rats; this may be due to neuroplasticity in this region of the brain.

Conflicts of interest

The authors have no conflicts of interest to declare.

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