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BRAIN RESEARCH

Highlights

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Amino acid tissue levels and $GABA_A$ receptor binding in the developing rat cerebellum following status epilepticus

Maria-Leonor López-Meraz[a,*], Luisa L. Rocha[b], Marta Miquel[c], Jenny C. Ortega[d], Cesar A. Perez-Estudillo[a], Luis I. García[a], M.E. Hernandez[a], J. Manzo[a]

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► Status epilepticus (SE) was induced using lithium–pilocarpine in developing rats. ► SE increased the tissue levels of taurine and alanine in the cerebellar hemispheres. ► SE did not modify amino acid tissue content in the cerebellar vermis. ► SE did not modify $GABA_A$ receptor binding in any lobule from the medial vermis. ► SE produced region-specific changes in the developing cerebellum.

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BRAIN RESEARCH

Research Report

Amino acid tissue levels and $GABA_A$ receptor binding in the developing rat cerebellum following status epilepticus

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ABSTRACT

Incidence of *status epilepticus* (SE) is higher in children than in adults and SE can be induced in developing rats. The cerebellum can be affected after SE; however, consequences of cerebellar amino acid transmission have been poorly studied. The goal of this study was to determine amino acid tissue concentration and $GABA_A$ receptor binding in the immature rat cerebellum after an episode of SE. Thirteen-day-old (P13) rat pups received intraperitoneal injections of lithium chloride (3 mEq/kg). Twenty hours later, on P14, SE was induced by subcutaneous injection of pilocarpine hydrochloride (60 mg/kg). Control animals were given an equal volume of saline subcutaneously. Animals were killed 24 h after SE induction, the cerebellum was quickly removed, and the vermis and hemispheres were rapidly dissected out on ice. Amino acid tissue concentrations in the vermis and hemispheres were evaluated by HPLC and fluorescent detection. $GABA_A$ receptor binding in the medial vermis was analyzed by *in vitro* autoradiography. SE increased the tissue levels of the inhibitory amino acids taurine (80%) and alanine (91%), as well as glutamine (168%) in the cerebellar hemisphere; no changes were observed in the vermis. SE did not modify $GABA_A$ receptor binding in any cerebellar lobule from the vermis. Our data demonstrate that SE produces region-specific changes in amino acid concentrations in the developing cerebellum.

1. Introduction

Status epilepticus (SE) is considered a non-self-limited type of epileptic seizure (Engel, 2006) and is characterized by an enduring epileptic state during which seizures are unremitting and tend to be self-perpetuating (Chen et al., 2007). Epidemiologic studies indicate that SE is more common in young children (DeLorenzo et al., 1995, 1996; Hauser, 1994). SE can be induced experimentally in developing rats using the lithium–pilocarpine model, which reproduces motor seizure manifestations and causes extensive neuronal injury in several brain areas (Lopez-Meraz et al., 2010; Sankar et al., 1992).

Participation of the cerebellum in seizures or epilepsy has been under debate for several years. Some reports suggest

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that cerebellar outflow pathways are seizure inhibitory (Dow et al., 1962; Miller et al., 1993; Rubio et al., 2011). Additionally, data from humans and experimental animal models show that SE can cause damage in the cerebellum (Crooks et al., 2000; Dam et al., 1984; Fujikawa et al., 2000; Leifer et al., 1991; Suga and Wasterlain, 1980); however, few studies have investigated the consequences of SE on the developing cerebellum. In this respect, it is known that SE reduces cerebellar weight and DNA synthesis in immature rats, effects that are related to a delay in maturation of behavioral milestones (Wasterlain, 1976). Recently, it has been reported that pentylenetetrazol-induced seizures in 10-day-old (P10) rat pups lead to loss of Purkinje cells and reduced cell proliferation in the cerebellum (Lomoio et al., 2011).

Amino acid transmitters are particularly abundant in the cerebellum (Plaitakis, 1992). Most synaptic inhibition in the cerebellum, beginning in the second week of life, is mediated by $GABA_A$ receptors (Brickley et al., 1996), which are abundant in the granule layer (Brickley et al., 1996; Fritschy and Panzanelli, 2011). It has been reported that during lithium–pilocarpine-induced SE in adult rats, amino acid levels can be modified, *e.g.*, aspartate concentration in the whole cerebellum decreases, whereas glutamine increases (Walton et al., 1990). Additionally, 30 min of hyperthermic seizures induced in P10 rats modifies tissue amino acid concentration in the cerebellum 24 h following convulsions, changes that include decreases in GABA, taurine, and alanine inhibitory amino acid levels and an increase in the concentration of the excitatory amino acid aspartate (González Ramírez et al., 2010). Thus, the purpose of this study was to determine amino acid concentrations and $GABA_A$ receptor binding in the cerebellar vermis and hemispheres after lithium–pilocarpine-induced SE in P14 rats.

2. Results

All animals injected with pilocarpine developed generalized motor seizures scored as stage 5 as well as SE. Latency to SE was 17 ± 0.7 min, and duration of behavioral SE was 4.7 ± 0.4 h.

2.1. *Tissue amino acid content*

In hemispheres, taurine (TAU, 80%; $t(8) = -2.886$, $p = 0.02$), alanine (ALA, 91%; $t(8) = -2.629$, $p = 0.03$), and glutamine (GLN, 168%; $t(8) = -2.881$, $p = 0.02$) displayed significantly increased levels following SE compared with control rats; no changes between SE and control groups were observed in the tissue concentration of glutamate (GLU, $t(8) = -1.715$; $p = 0.13$), aspartate (ASP, $t(8) = -1.29$; $p = 0.22$), GABA ($t(8) = -1.743$; $p = 0.12$), and glycine (GLY, $t(8) = -1.779$; $p = 0.11$). In the vermis, no difference was detected in the concentration of any amino acid analyzed following seizures as compared with the control group (Fig. 1).

2.2. *$GABA_A$ receptor binding*

Apparent ^{3}H-muscimol binding was detected in the granule layer of all lobules from the medial vermis. Low $GABA_A$ receptor levels were also observed in the molecular layer (data not

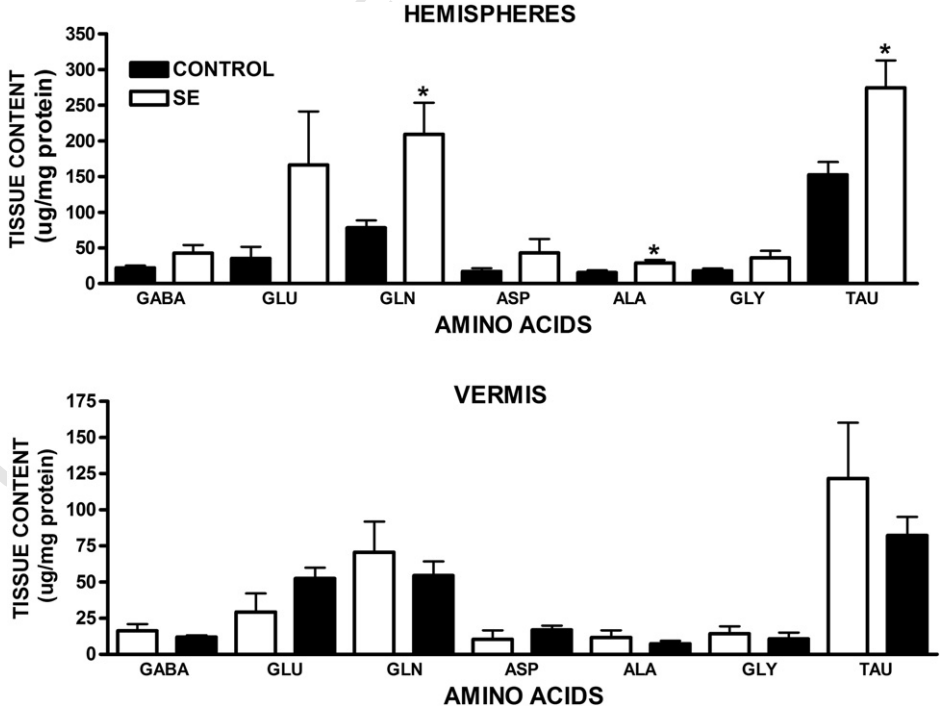

Fig. 1 – Amino acid tissue content (μg/mg protein) in hemispheres (upper panel) and the vermis (bottom panel) 24 h after SE (white bars) or in age-matched controls (black bars). Abbreviations: GABA, γ-aminobutyric acid; GLU, glutamate; GLN, glutamine; ASP, aspartate; ALA, alanine; GLY, glycine; TAU, taurine. The graphs represent the mean ± S.E.M. (n = 5 per group) *$p < 0.05$ *vs.* control; Student's t-test.

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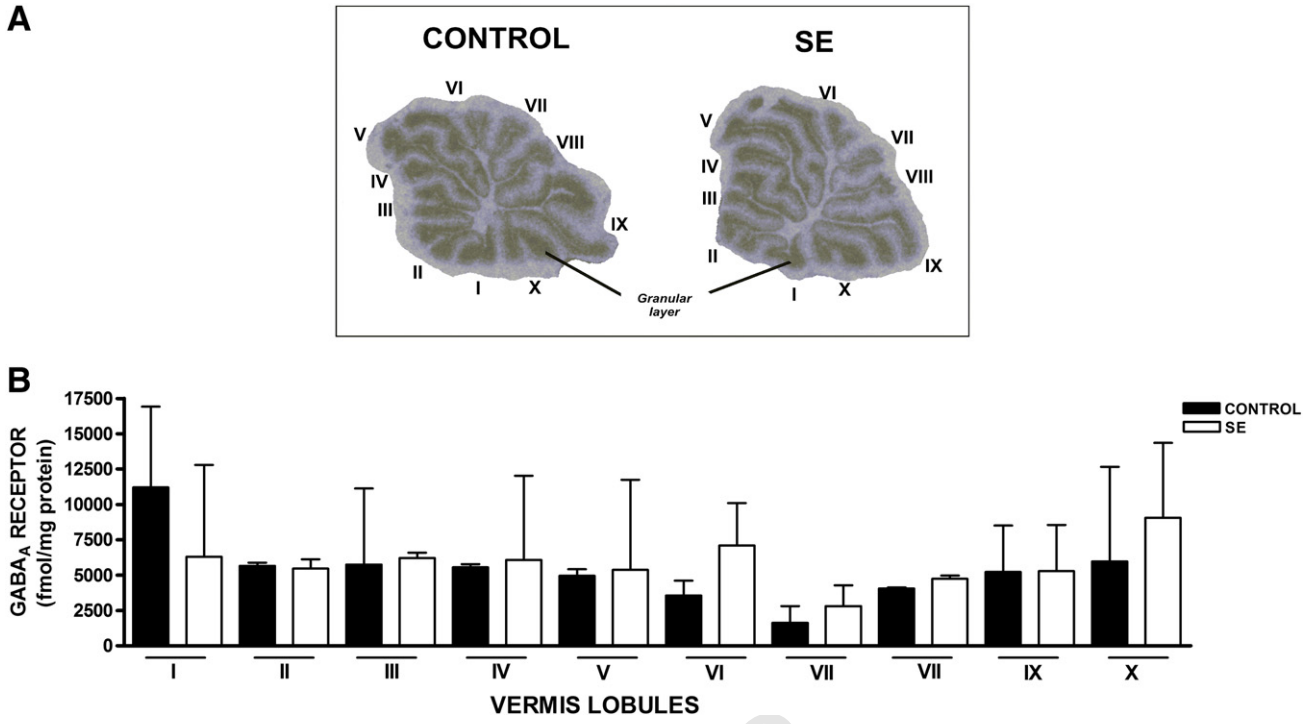

Fig. 2 – Representative distribution of $GABA_A$ receptors labeled with ^{3}H-muscimol in cerebellar sections at the medial vermis from a control rat (left panel) and an SE rat (right panel) (A). High receptor binding appears as black or dark gray color, whereas white color indicates low receptor binding. Cerebellar lobules are numbered from I to X. $GABA_A$ receptor levels (fmol/mg protein) in cerebellar lobules from the medial vermis (B). Black bars represent control animals, and white bars correspond to animals 24 h after SE. Data are represented as the median ± interquartile range (n = 4 per group). No statistical differences were found between experimental groups when compared using the Mann–Whitney *U* test.

shown here), but no obvious ^{3}H-muscimol binding was detected in the Purkinje layer (Fig. 2). SE did not significantly modify the $GABA_A$ receptor levels in the granule layer of each cerebellar lobule compared to those from control animals ($p > 0.05$; Fig. 2). Total cerebellar $GABA_A$ receptor binding was similar in the vermis of control (5909 ± 1173 fmol/mg protein) and SE (6608 ± 4234 fmol/mg protein) groups ($U = 9$, $p = 0.886$).

3. Discussion

In this study, we observed enhanced concentrations of ALA, TAU, and GLN, but not GLU, ASP, GABA, or GLY in the cerebellar hemispheres 24 h after SE induction in P14 rats. We found no changes in any amino acid level in the vermis. In addition, our results show that $GABA_A$ receptor binding is not altered in any lobule of the medial vermis one day after SE. These data suggest that SE induces particular neurochemical changes in the immature cerebellum and that these effects are region-specific.

The amino acids TAU and ALA have inhibitory effects on neuronal activity (Horikoshi et al., 1988; O'Byrne and Tipton, 2000). One explanation for their increase in our SE model could be that they are part of postictal neurochemical changes launched to avoid a new seizure. It is interesting that TAU, which is important in Purkinje neurons (Terauchi et al., 1998), may act as neuromodulator or transmitter to augment inhibitory outflow to decrease motor responses mediated by the cerebellum after SE, either alone or in coordination with GABA, similar to that observed in the hippocampus after medial septal lesions (Rodriguez et al., 2005). The increased postictal GLN concentration may be the result of augmented GLU synthesis during SE onset as observed in adult rats (Walton et al., 1990); however, additional quantification of amino acids during developmental SE is necessary to support this hypothesis.

A previous report showed that 30 min of hyperthermic seizures in P10 rats increased $GABA_A$ and benzodiazepine receptor binding (which is coupled to the $GABA_A$ complex) in cortex, hippocampus, amygdala, thalamus, and other mesencephalic structures 24 h following seizures (González Ramírez et al., 2007). Similarly, Rocha et al. (2007) showed that one week following lithium–pilocarpine-induced SE in P12 rats, benzodiazepine receptor levels increased in cortical structures and amygdaloid nuclei; however, the cerebellum was not evaluated. Our study showed strong ^{3}H-muscimol binding in the developing vermis, supporting previous investigations showing the abundance of this receptor in the granule layer (Fritschy and Panzanelli, 2011). However, SE did not modify $GABA_A$ binding in any lobule of the cerebellar cortex. This effect may result from the elevated concentration of $GABA_A$ receptors in the cerebellum of two-week-old rats, such that SE no longer modifies this variable or the high plasticity of the GABAergic system (including $GABA_A$ receptor subunits) in

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the developing cerebellum (Takayama, 2005; Thompson and Stephenson, 1994). Differences between our study and other reports about amino acid biochemistry and $GABA_A$ receptor radiolabeling in the cerebellum could be due to the age of the rat, the duration of seizures (considering that in our study we tried to mimic severe SE that was not stopped with any drug), the model employed to induce SE (hyperthermic seizures are not the same as SE), the time post-seizures at which amino acids were evaluated (ictal *vs.* postictal), and the fact that we did not evaluate the whole cerebellum but separated the vermis from hemispheres.

Differences between the cerebellar vermis and hemispheres observed in this study may be explained by considering that these regions have different efferent and afferent pathways (Voogd, 2004). Of note, cortical projections to the cerebellum and *vice versa* involve mainly the hemispheres (Baker et al., 2001; Ramnani, 2006). This fact is important because the neocortex is involved in seizure generation or is affected by SE in the developing rat (Cavalheiro et al., 1997; Suchomelova et al., 2006). In conclusion, this study supports our hypothesis that SE affects the developing cerebellum, modifying TAU, ALA, and GLN amino acid concentrations in hemispheres but not in the vermis. $GABA_A$ receptors, at least in the medial vermis, are not modified under these conditions.

4. Experimental procedures

4.1. *Animals*

Wistar rat pups of both sexes (Instituto de Neuroetología, Universidad Veracruzana, Mexico) were used. The day of birth was considered day 0. Pups were housed with their dams with 12 h light–dark cycles (7 am-7 pm) and had free access to food and water. Experiments were approved by a Committee of Graduate Program in Neuroethology, Instituto de Neuroetología, Universidad Veracruzana to minimize the number of animals used and their suffering. Studies were conducted in accordance with Mexican guidelines on the care and use of laboratory animals (NOM-062-ZOO-1999).

4.2. *Induction of SE*

P13 rat pups were given intraperitoneal injections of lithium chloride (3 mEq/kg; #L-0505 Sigma), and 20 h later, SE was induced with subcutaneous injection of pilocarpine hydrochloride (60 mg/kg; #P6503 Sigma) as described previously (Sankar et al., 1992). Control rats were given an equal volume of lithium chloride and saline instead of the convulsant. Behavioral motor seizures were carefully monitored by an experienced analyst and scored according to a slightly modified Racine scale (1972): (0 = behavioral arrest; 1 = face clonus; 2 = head nodding; 3 = forelimb clonus; 4 = forelimb clonus and rearing; 5 = forelimb clonus with rearing and falling). Only animals reaching SE, defined as near continuous seizure activity lasting over 30 min (Wasterlain and Chen, 2006), were included in the study. After SE, pups received 1 ml isotonic 5% dextrose in saline solution subcutaneously to avoid dehydration without stressing the cardiovascular system. After the cessation of seizures, pups were placed back with their mothers (approximately 6 h to avoid cannibalism); time of separation from the mother was strictly controlled and was similar in control and SE groups. There was no mortality in this study.

4.3. *Tissue processing*

For analysis of amino acid concentrations, rats were anesthetized with pentobarbital 24 h after SE or saline injection (n=5 per group), and cerebella were quickly removed and divided into the vermis and hemispheres (left and right). For autoradiography experiments, all rats (n=4 per group) were rapidly killed by decapitation (following previously reported protocols and considering that anesthesia may affect receptor binding) 24 h post-SE or saline, cerebella were quickly removed, and the vermis was obtained. $GABA_A$ receptor binding studies were carried out exclusively at the medial vermis. Tissues were frozen in liquid nitrogen and stored at –86 °C for posterior autoradiography and chromatography experiments.

4.4. *Determination of amino acid tissue levels with High-Performance Liquid Chromatography (HPLC)*

Cerebellar tissue was homogenized in 0.1 M perchloric acid containing 4 mM sodium metabisulfite solution (30 μl per 10 mg of tissue; Peat and Gibb, 1983). The resulting homogenate was centrifuged at 10,000 rpm at 4 °C for 20 min, and the residual pellet was separated from the supernatant, which was also filtered through a syringe Millex®-HN filter (0.45 μm pore). Pellet and filtered supernatant were stored separately in Eppendorf tubes at –86 °C until protein and amino acid analyses, respectively. Concentration of amino acids was measured using precolumn derivatization with o-phthaldehyde (OPA) and fluorescence detection as described by Kendrich et al. (1988). Derivatization was performed by mixing 20 μl filtered supernatant with 6 μl OPA and injecting this mixture into the solvent stream of the HPLC system 2 min later. Separation of OPA-amino acids was carried out on a reversed-phase 3.9×150-mm column (Nova-Pack, 4 μm, C18, Waters®) at 35 °C using a binary gradient system [mobile phase A: 38.74 mM sodium acetate dissolved in 90% milli-Q water and 10% methanol, pH 5.75; and mobile phase B: buffer containing 20% solution A and 80% methanol, pH 6.75] at a flow rate of 0.5 ml/min. Fluorometric detection was performed with a Waters® model 474 detector at excitation and emission wavelengths of 360 and 450 nm, respectively. This procedure allowed the quantification of GABA, GLU, GLN, ASP, ALA, GLY, and TAU levels by linear regression using external standards (Sigma). Protein determination was carried out using the residual pellet according to a modified version of Bradford's method (Bradford, 1976). Amino acid tissue content was expressed as μg/mg protein.

4.5. *$GABA_A$ receptor binding by in vitro autoradiography*

Frozen sagittal sections of 20 μm at the level of the medial vermis were cut on a cryostat, thaw-mounted onto gelatin-coated slides, and stored at –86 °C until the day of incubation. *In vitro* autoradiography was performed as described previously (González Ramírez et al., 2007) on parallel sections to label $GABA_A$ receptors. Briefly, cerebellar sections were pre-washed

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for 30 min at room temperature in 50 mM Tris HCl-citrate buffer pH 7.4 (both from Sigma) to remove endogenous ligands. Then, they were incubated in a solution containing 10 nM muscimol-[methylene-^{3}H(N)] (NET, S.A. 20 Ci/mmol), a competitive $GABA_A$ receptor agonist, in the presence or absence of 10 μM GABA (a saturating concentration, Sigma) for 45 min at 4 °C. Incubation was completed with two consecutive buffer washes (1 min each at 4 °C). Finally, the slides were rinsed (3 s) in distilled water at 4 °C, and the sections were quickly dried under a gentle stream of cold air. The slides were arranged in X-ray cassettes together with tritium standards (Amersham) and exposed to radioactivity-sensitive film (Biomax-MR, Kodak) at room temperature for 10 weeks. Films were developed using developer D19 (Kodak) and fixer at room temperature. Optical densities of the autoradiograms were determined using a video-computer enhancement program (JAVA Jandel Video Analysis Software). The optical density of the standards was used to determine tissue radioactivity values for the accompanying tissue sections and to convert them to fmol/mg protein. Cerebella lobules at the medial vermal level were identified according to the stereotaxic atlas of the developing rat brain (Sherwood and Timiras, 1970). $GABA_A$ binding was analyzed at the granular layer and carried out in lobules I–X. For each lobule, 10 optical density readings were taken from three sections and averaged. Receptor binding was expressed as fmol/mg protein.

4.6. *Statistical analysis*

Data for each amino acid tissue concentration from the vermis and hemispheres were analyzed with a Student's t-test. Results from $GABA_A$ receptor binding for each vermis lobule as well as for the whole cerebellum were analyzed with a Mann–Whitney *U* test. Sigma Stat version 3.5 (Systat Sofware Inc.) was used for the statistical analysis, and $p<0.05$ was considered significant. Data are presented as the mean±S.E.M. (seizure behavior and amino acids) or median±interquartile range ($GABA_A$ receptor binding).

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