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Highlights

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

Brain Research xxx (2012) xxx–xxx

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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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Research Report

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

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ARTICLE INFO

Article history:

Accepted 17 December 2011

Keywords:

Status epilepticus
Developing rat
Cerebellum
GABA_A receptor
Amino acids

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.

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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). Epidemiological studies indicate that SE is more common in young chil-

dren (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 ³H-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

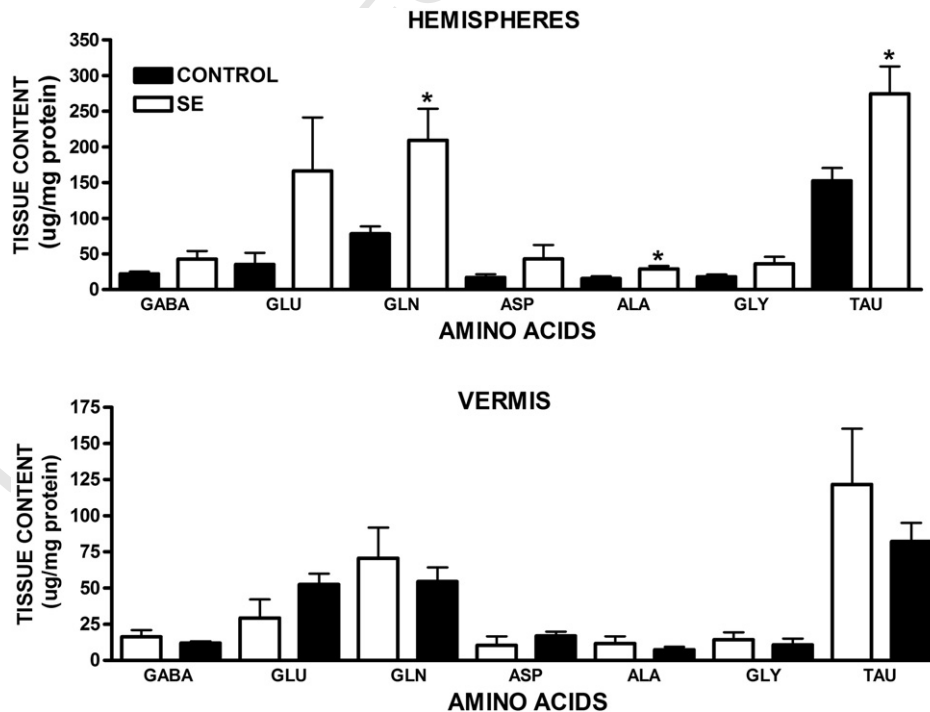


Fig. 1 – Amino acid tissue content ($\mu\text{g}/\text{mg}$ protein) in hemispheres (upper panel) and the vermis (bottom panel) 24 h after SE (white bars) or in age-matched controls (black bars). Abbreviations: GABA, γ -aminobutyric acid; GLU, glutamate; GLN, glutamine; ASP, aspartate; ALA, alanine;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

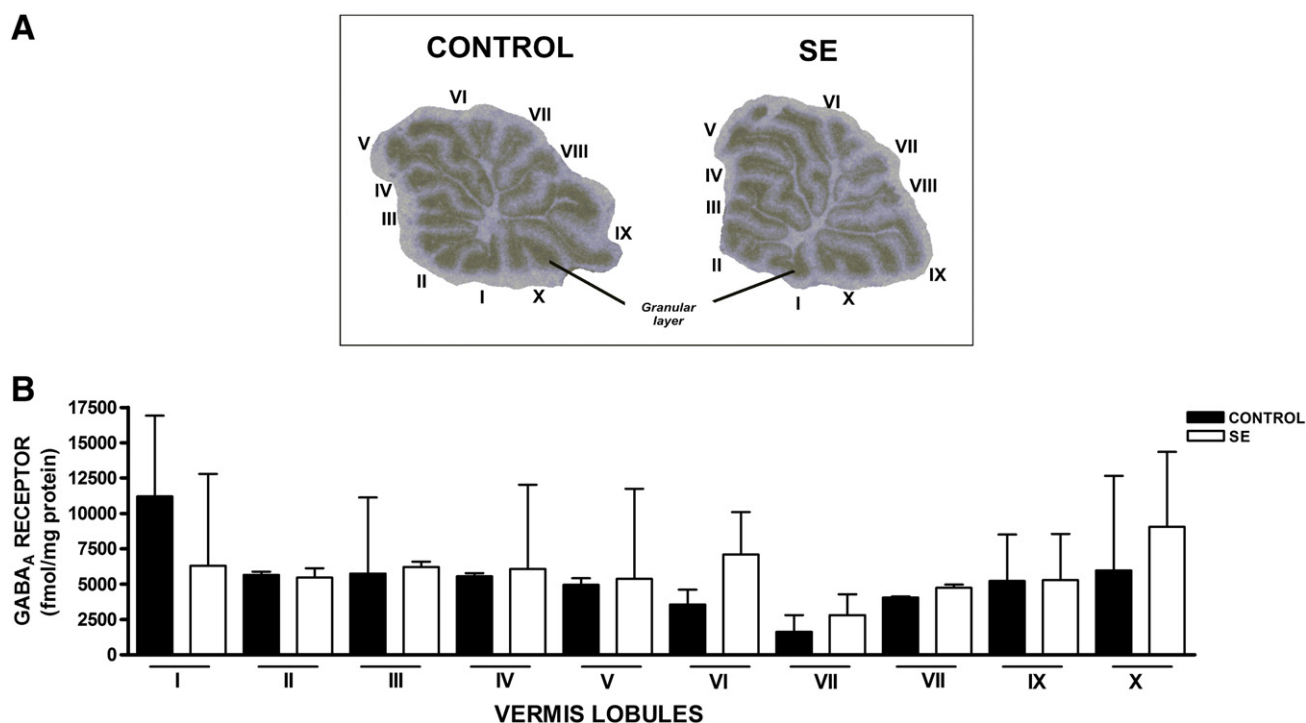


Fig. 2 – Representative distribution of GABA_A receptors labeled with ³H-muscimol in cerebellar sections at the medial vermis from a control rat (left panel) and an SE rat (right panel) (A). High receptor binding appears as black or dark gray color, whereas white color indicates low receptor binding. 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.

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

120 3. Discussion

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

130 The amino acids TAU and ALA have inhibitory effects on
 131 neuronal activity (Horikoshi et al., 1988; O'Byrne and Tipton,
 132 2000). One explanation for their increase in our SE model
 133 could be that they are part of postictal neurochemical
 134 changes launched to avoid a new seizure. It is interesting
 135 that TAU, which is important in Purkinje neurons (Terauchi
 136 et al., 1998), may act as neuromodulator or transmitter to

137 augment inhibitory outflow to decrease motor responses me-
 138 diated by the cerebellum after SE, either alone or in coordina-
 139 tion with GABA, similar to that observed in the hippocampus
 140 after medial septal lesions (Rodríguez et al., 2005). The in-
 141 creased postictal GLN concentration may be the result of
 142 augmented GLU synthesis during SE onset as observed in
 143 adult rats (Walton et al., 1990); however, additional quantifi-
 144 cation of amino acids during developmental SE is necessary
 145 to support this hypothesis.

146 A previous report showed that 30 min of hyperthermic sei-
 147 zures in P10 rats increased GABA_A and benzodiazepine recep-
 148 tor binding (which is coupled to the GABA_A complex) in cortex,
 149 hippocampus, amygdala, thalamus, and other mesencephalic
 150 structures 24 h following seizures (González Ramírez et al.,
 151 2007). Similarly, Rocha et al. (2007) showed that one week fol-
 152 lowing lithium-pilocarpine-induced SE in P12 rats, benzodiaz-
 153 epine receptor levels increased in cortical structures and
 154 amygdaloid nuclei; however, the cerebellum was not evaluat-
 155 ed. Our study showed strong ³H-muscimol binding in the de-
 156 veloping vermis, supporting previous investigations showing
 157 the abundance of this receptor in the granule layer (Fritschy
 158 and Panzanelli, 2011). However, SE did not modify GABA_A
 159 binding in any lobule of the cerebellar cortex. This effect
 160 may result from the elevated concentration of GABA_A recep-
 161 tors in the cerebellum of two-week-old rats, such that SE no
 162 longer modifies this variable or the high plasticity of the
 163 GABAergic system (including GABA_A receptor subunits) in

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

175 Differences between the cerebellar vermis and hemi-
176 spheres observed in this study may be explained by consider-
177 ing that these regions have different efferent and afferent
178 pathways (Voogd, 2004). Of note, cortical projections to the cer-
179 ebellum and *vice versa* involve mainly the hemispheres (Baker
180 et al., 2001; Ramnani, 2006). This fact is important because the
181 neocortex is involved in seizure generation or is affected by SE
182 in the developing rat (Cavalheiro et al., 1997; Suchomelova et
183 al., 2006). In conclusion, this study supports our hypothesis
184 that SE affects the developing cerebellum, modifying TAU,
185 ALA, and GLN amino acid concentrations in hemispheres but
186 not in the vermis. GABA_A receptors, at least in the medial ver-
187 mis, are not modified under these conditions.

188 4. Experimental procedures

190 4.1. Animals

191 Wistar rat pups of both sexes (Instituto de Neuroetología, Uni-
192 versidad Veracruzana, Mexico) were used. The day of birth
193 was considered day 0. Pups were housed with their dams
194 with 12 h light-dark cycles (7 am-7 pm) and had free access
195 to food and water. Experiments were approved by a Commit-
196 tee of Graduate Program in Neuroethology, Instituto de Neu-
197 roetología, Universidad Veracruzana to minimize the
198 number of animals used and their suffering. Studies were con-
199 ducted in accordance with Mexican guidelines on the care and
200 use of laboratory animals (NOM-062-ZOO-1999).

201 4.2. Induction of SE

202 P13 rat pups were given intraperitoneal injections of lithium
203 chloride (3 mEq/kg; #L-0505 Sigma), and 20 h later, SE was in-
204 duced with subcutaneous injection of pilocarpine hydrochlo-
205 ride (60 mg/kg; #P6503 Sigma) as described previously
206 (Sankar et al., 1992). Control rats were given an equal volume
207 of lithium chloride and saline instead of the convulsant. Be-
208 havioral motor seizures were carefully monitored by an experi-
209 enced analyst and scored according to a slightly modified
210 Racine scale (1972): (0 = behavioral arrest; 1 = face clonus; 2 =
211 head nodding; 3 = forelimb clonus; 4 = forelimb clonus and
212 rearing; 5 = forelimb clonus with rearing and falling). Only an-
213 imals reaching SE, defined as near continuous seizure activity
214 lasting over 30 min (Wasterlain and Chen, 2006), were includ-
215 ed in the study. After SE, pups received 1 ml isotonic 5% dex-
216 trose in saline solution subcutaneously to avoid dehydration
217 without stressing the cardiovascular system. After the cessa-
218 tion of seizures, pups were placed back with their mothers

(approximately 6 h to avoid cannibalism); time of separation 219
from the mother was strictly controlled and was similar in 220
control and SE groups. There was no mortality in this study. 221

222 4.3. Tissue processing

For analysis of amino acid concentrations, rats were anesthe- 223
tized with pentobarbital 24 h after SE or saline injection (n=5 224
per group), and cerebella were quickly removed and divided 225
into the vermis and hemispheres (left and right). For autoradi- 226
ography experiments, all rats (n=4 per group) were rapidly 227
killed by decapitation (following previously reported protocols 228
and considering that anesthesia may affect receptor binding) 229
24 h post-SE or saline, cerebella were quickly removed, and 230
the vermis was obtained. GABA_A receptor binding studies 231
were carried out exclusively at the medial vermis. Tissues 232
were frozen in liquid nitrogen and stored at -86 °C for posterio- 233
r autoradiography and chromatography experiments. 234

235 4.4. Determination of amino acid tissue levels with 236 High-Performance Liquid Chromatography (HPLC)

Cerebellar tissue was homogenized in 0.1 M perchloric acid 237
containing 4 mM sodium metabisulfite solution (30 µl per 238
10 mg of tissue; Peat and Gibb, 1983). The resulting homoge- 239
nate was centrifuged at 10,000 rpm at 4 °C for 20 min, and 240
the residual pellet was separated from the supernatant, 241
which was also filtered through a syringe Millex®-HN filter 242
(0.45 µm pore). Pellet and filtered supernatant were stored 243
separately in Eppendorf tubes at -86 °C until protein and 244
amino acid analyses, respectively. Concentration of amino 245
acids was measured using precolumn derivatization with o- 246
phthaldehyde (OPA) and fluorescence detection as described 247
by Kendrich et al. (1988). Derivatization was performed by 248 Q2
mixing 20 µl filtered supernatant with 6 µl OPA and injecting 249
this mixture into the solvent stream of the HPLC system 250
2 min later. Separation of OPA-amino acids was carried out 251
on a reversed-phase 3.9×150-mm column (Nova-Pack, 4 µm, 252
C18, Waters®) at 35 °C using a binary gradient system [mobile 253
phase A: 38.74 mM sodium acetate dissolved in 90% milli-Q 254
water and 10% methanol, pH 5.75; and mobile phase B: buffer 255
containing 20% solution A and 80% methanol, pH 6.75] at a 256
flow rate of 0.5 ml/min. Fluorometric detection was performed 257
with a Waters® model 474 detector at excitation and emission 258
wavelengths of 360 and 450 nm, respectively. This procedure 259
allowed the quantification of GABA, GLU, GLN, ASP, ALA, 260
GLY, and TAU levels by linear regression using external stan- 261
dards (Sigma). Protein determination was carried out using 262
the residual pellet according to a modified version of Brad- 263
ford's method (Bradford, 1976). Amino acid tissue content 264
was expressed as µg/mg protein. 265

266 4.5. GABA_A receptor binding by *in vitro* autoradiography

Frozen sagittal sections of 20 µm at the level of the medial ver- 267
mis were cut on a cryostat, thaw-mounted onto gelatin-coated 268
slides, and stored at -86 °C until the day of incubation. *In vitro* 269
autoradiography was performed as described previously 270
(González Ramírez et al., 2007) on parallel sections to label 271
GABA_A receptors. Briefly, cerebellar sections were pre-washed 272

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-³H(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 Software 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).

Acknowledgments

This study was funded by CONACyT grants 106402 (MLLM), 98386 (LR), scholarship 161511 (JCO), and PROMEP-SEP grant PROMEP/103.5/10/5006 (MLLM, PTC-474). We thank the excellent technical assistance of Leticia Neri Bazán, B.Sc.

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