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Amino acid levels in some lethargic mouse brain areas before and after pentylenetetrazole kindling

Grazia De Luca[a], Rosa Maria Di Giorgio[a], Salvatore Macaione[a], Pina Rita Calpona[a], Santa Costantino[a], Eugenio Donato Di Paola[b], Nicola Costa[c,d], Domenicantonio Rotiroti[c,d], Guido Ferreri Ibbadu[b], Emilio Russo[b], Giovambattista De Sarro[b,*]

[a]*Department of Biochemical, Physiological and Nutritional Sciences, School of Medicine, University of Messina, AOU "G. Martino" via Consolare Valeria, 98125 Messina, Italy*

[b]*Unit of Pharmacology, Department of Experimental and Clinical Medicine "G. Salvatore", School of Medicine, University "Magna Graecia" of Catanzaro, Italy*

[c]*Institute of Neurological Science of CNR, Section of Catanzaro, Italy*

[d]*Department of Pharmacobiological Science, Faculty of Pharmacy, University "Magna Graecia" of Catanzaro, Complesso "Nini Barbieri", Roccelleta di Borgia, Catanzaro, Italy*

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Abstract

Genetic animal models have contributed significantly to our understanding of epilepsy causes. Lethargic mice are considered a valid model of absence epilepsy, which have been shown to possess behavioral, electrographic and pharmacological profiles similar to those of humans with absence epilepsies. Single gene mutations that comprise the β_4 subunit of voltage-sensitive Ca^{2+} channels underlie the spontaneous discharges of the absence, non-convulsive seizures of lethargic mice. There are no available data concerning how the mutant channels actually behave at terminals in response to chemical activation by subconvulsant stimulation with pentylenetetrazole.

In this study, we found no significant difference in the convulsive dose 50 between lethargic and control mice. Lethargic mice showed a more rapid development of kindling to pentylenetetrazole than control animals. No significant differences were observed between the groups of mice rechallenged with pentylenetetrazole 30 or 60 days after the end of the chronic treatment. Marked differences in brain amino acid levels were found between the two strains of mice in basal conditions and after kindling. In conclusion, our results indicate that lethargic mice show a range of biochemical and behavioral changes, correlated in particular with a higher susceptibility to develop kindled seizures.

Keywords: Epilepsy; Pentylenetetrazole kindling; Neuroactive amino acids; Absence seizures; Lethargic mice

1. Introduction

Genetic animal models have contributed significantly to our understanding of the causes of epilepsies (Burgess and Noebels, 1999). Lethargic (*lh/lh*) mice are considered a valid genetic model of absence epilepsy, which have been shown to possess behavioral, electrographic and pharmacological profiles similar to those of humans with absence epilepsies (Hosford et al., 1992, 1995). Single gene mutations that comprise the β_4 subunit of voltage-sensitive Ca^{2+} channels underlie the spontaneous discharges of the absence, non-convulsive seizures of *lh/lh* mice. β subunits have been shown to attenuate G-protein coupled inhibition of Ca^{2+} channels and to modulate the conductance through the α_1 subunit (Campbell et al., 1995). Other evidences indicate that an increase in β_3 subunit expression might

Abbreviations: PTZ, pentylenetetrazole; *lh/lh*, lethargic; WT, wild type/control mice; s.c., subcutaneously; CX, cortex; HI, hippocampus; CB, cerebellum; BS, brain stem; DE, diencephalon; AA, amino acid.

* Corresponding author. Dipartimento di Medicina Sperimentale e Clinica "G. Salvatore", Policlinico "Mater Domini", Università degli Studi di Catanzaro, Via T. Campanella, 115, 88100 Catanzaro (CZ), Italy. Tel.: +39 961 712323; fax: +39 961 774424.

E-mail address: desarro@unicz.it (G. De Sarro).

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compensate for the defective β_4 subunits of *lh/lh* mice, except in some brain areas, including the thalamus and neocortex (Burgess et al., 1999; Lin et al., 1999a). Genetic analysis of the *lethargic* mutation indicated that the locus encodes a truncated cytoplasmic β_4 subunit, resulting in the loss of functional $\alpha_1-\beta_4$ interactions (Burgess et al., 1997). β_4 subunits interact with both α_{1A} and α_{1B} transmembrane subunits playing a key role in channel assembly and localization, and possess unique modulatory sites not shared by other β subunits (Walker and De Waard, 1998). The loss of β_4 subunits in *lh/lh* mutants alters the function of both P/Q- and N-type Ca^{2+} channels (Caddick et al., 1999). Given the pivotal role of Ca^{2+} channels in controlling neurotransmitter release, defects in the structure, localization and modulation of presynaptic Ca^{2+} channels are expected to result in aberrant synaptic signalling leading to various patterns of neuronal network dysfunctions.

There are no available data concerning how the mutant channels actually behave at terminals in response to chemical activation by subconvulsant stimulation with pentylenetetrazole (PTZ), a compound exerting its convulsant effects by impairment of $GABA_A$-mediated neurotransmission (Corda et al., 1991; McNamara et al., 1989). In this study, we compared the convulsive dose 50 (CD_{50}) between *lh/lh* mice and the seizure free littermates (WT). Furthermore, we examined possible differences in the ability of the two strains in developing kindling induced by injections of PTZ subconvulsant doses and the differences in amino acid concentrations in different brain areas before and after the development of the kindling status.

2. Materials and methods

2.1. Animals

Lethargic (*lh/lh*) mice (B6EiC3Sn *a*/A-*Cacnb4*^{lh}) lacking the β_4 subunit of voltage-activated Ca^{2+} channels were originally obtained from The Jackson Laboratories (Bar Harbor, Maine, USA) and then colonies were maintained at the Faculty of Pharmacy, University of Catanzaro (Italy). As control animals, their seizure free littermates (WT) have been used. Animals were maintained under environmentally controlled conditions (7 a.m./7 p.m. light/dark cycle, 22–24 °C, with food and water available ad libitum).

The mice used at the beginning of this study were from 63 to 80 days old and weight 24–30 g, male only. Procedures involving animals and their care were conducted in conformity with national and international laws and policies (EEC Council Directive of 24 November 1986 (86/609EEC). All animal experiments were carried out according to the NIH animal care guidelines (NIH Publication N. 80–23). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

2.2. Pentylenetetrazole-induced seizure

Both strains of mice were injected s.c. with various doses of PTZ (30–90 mg/kg). In particular, animals were divided in groups of at least 10 mice and treated with one of the four doses of PTZ (30, 50, 70 and 90 mg/kg). After PTZ administration, animals were placed in isolated cages ($30 \times 30 \times 30$ cm) and observed for the following 30 min. A threshold convulsion was considered an episode of clonic spasms lasting for at least 5 s. Absence of this threshold convulsion over the 30 min observation period indicated that the animal was protected from the convulsant-induced seizures (Swinyard and Woodhead, 1982). Each animal was used only once.

2.3. Development of kindling by PTZ

Kindling was produced by PTZ (30 mg/kg) injected s.c. every other day for 9 consecutive weeks in the morning between 9:00 and 11:00. In each experiment, the mice were placed in a Plexiglas box ($30 \times 30 \times 30$ cm) and observed for the following 120 min for the incidence and onset of convulsions. The intensity of the seizure response was scored on the following scale: 0=no response; 1=mouth and facial jerks; 2=nodding or myoclonic body jerks; 3=forelimb clonus; 4=rearing, falling down, hindlimb clonus and forelimb tonus; and 5=tonic extension of hindlimb, status epilepticus and/or death (De Sarro et al., 1999). The maximum response was recorded for each animal. The mice in each group were also observed for latency of seizure response (De Sarro et al., 1999). Any mouse that convulsed in response to the kindling treatment on the first day was excluded from the study. Mice were considered fully kindled when exhibiting three times stage 4 seizures, and then the treatment was discontinued. In order to have comparable withdrawal periods across treatments, on the same day, the treatment of one mouse in each other experimental group was discontinued. The mice that not fulfilled the kindling criteria after 9 weeks of repeated treatment were considered resistant and not used in the subsequent seizure studies. The mice whose treatment was discontinued after reaching the kindling criteria were assigned with the score of their last week of treatment for each of the remaining weeks in the calculation of the results.

2.4. Measurement of amino acid levels

Amino acid levels were determined in brain stem (BS), cerebellum (CB), cortex (CX), diencephalon (DE) and hippocampus (HI) of *lh/lh* and C57BL/6J mice at 60 days of age or 10 days after the development of kindling. Animals were decapitated without anesthesia, brains quickly removed and dissected to obtain the desired brain areas which were frozen at −80 °C until assayed. On the day of analysis, the samples were thawed and homogenized

in 0.1N HCl containing methionine sulfone and nor-leucine as internal standards. After centrifugation for 15 min at 1800 ×*g* at 4 °C, the resulting supernatants were deproteinized by ultrafiltration and 50 µl aliquots of ultrafiltrate were dried. Measurements of amino acid concentrations were performed by RP-HPLC using the Pico-Tag method (Waters) according to manufacturer's specifications (Cohen et al., 1989). Amino acids were derivatized with phenylisothiocyanate and the phenylthiocarbamyl amino acid derivatives were separated on the C_{18} Pico-Tag physiological free amino acid column [300 × 3.9 mm (i.d.)], using a stated binary gradient of Waters eluents 1 and 2 at a flow rate of 1.0 ml/min.

2.5. Drugs

Pentylenetetrazole was purchased from Sigma (St. Louis, MO, U.S.A.). For systemic injection (0.1 ml/10 g of body weight of the mouse), PTZ was given subcutaneously (s.c.) as a freshly prepared solution in sterile saline (0.9% NaCl).

2.6. Statistical analysis

The maximum response for each mouse was recorded. Incidence of the seizure phases in *lh/lh* and WT mice was statistically compared using Fisher's exact probability test (data not shown in Tables). The percentages of animals exhibiting clonic or tonic seizures following PTZ administration were plotted against the corresponding doses by a computer construction of the dose–effect curves for calculation of CD_{50} (±95% confidence limits). The CD_{50} values for each group were calculated using a computer programme (SAS/STAT) of the method of Litchfield and Wilcoxon (1949). At least 32 animals were used to calculate each CD_{50} value. Statistical comparisons between PTZ-kindled mice were assessed using the Mann–Whitney *U* test to compare median seizure score data from the different groups. The delay of the onset of seizures was evaluated using a two way analysis of variance (ANOVA) followed by Bonferroni's corrected Student's *t*-test. The Student's *t*-test of the SAS statistical package for personal computer was used to analyze biochemical data (SAS Institute Inc, 1987). All tests were used two sided and $P<0.05$ was considered significant.

3. Results

3.1. Pentylenetetrazole-induced seizures

PTZ is a chemoconvulsant drug which exerts its activity impairing $GABA_A$-mediated neurotransmission (Owens and Kriegstein, 2002; Russo et al., 2004). No significant difference in the CD_{50} values between *lh/lh* and WT mice was observed. In particular, the CD_{50} values (±95% confidence limits) calculated following s.c. injection of PTZ were 53.86 (42.89–67.63) for *lh/lh* mice and 53.67 (42.21–68.26) for control mice.

3.2. Development of pentylenetetrazole kindling

The data shown in Fig. 1A represent the effects of treatment with a subconvulsant dose (30 mg/kg) of PTZ every other day on the development of kindled convulsions in the two strains of mice used. The data indicate that the development of kindled convulsions was directly proportional and cumulative with repeated exposure to PTZ. Fig. 1B illustrates the number of PTZ injection necessary to develop a kindled convulsion (phase 4, appearance of hind limb clonus and forelimb tonus). *Lh/lh* mice showed a more rapid development of kindling to PTZ during the 9 weeks of treatment than WT, and consequently, the number of PTZ injections required to develop a phase 4 kindled convulsion was significantly lower ($P<0.05$).

There was no significant difference between the group of animals in response to the first injection of PTZ and the same applied for the animals that did not reach the kindling status after 9 weeks. In particular, none of the animals

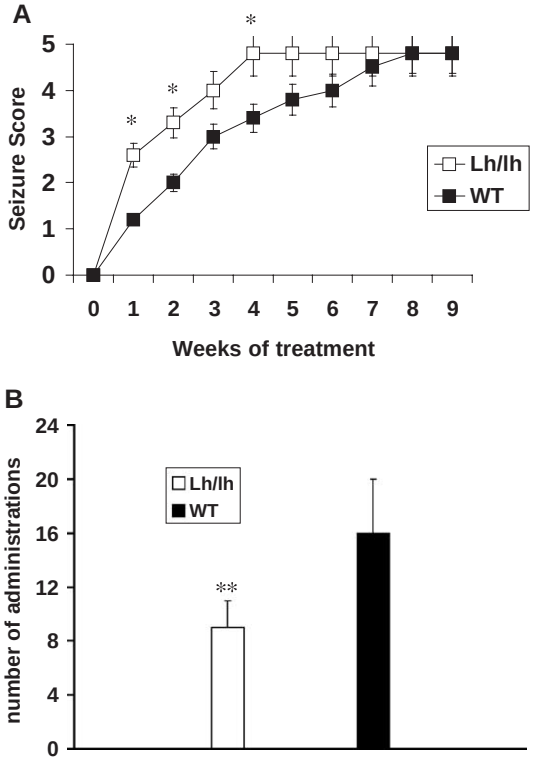

Fig. 1. Development of PTZ kindling. Effects of the repeated administration of PTZ on the manifestation of kindling during every other day treatment with PTZ (30 mg/kg, s.c.). (A) Ordinate shows the seizure score, abscissae show the weeks of repeated treatment. (B) Ordinate shows the number of administration needed to reach stage 4 seizures. Significant differences between the two strains after the development of kindling are denoted as $*P<0.05$, $**P<0.01$.

convulsed on the first administration and only 1/20 and 2/20, for WT and *lh/lh* mice, respectively, did not reach the kindling status after 9 weeks.

3.3. Amino acid levels in basal conditions

A statistical comparison between the amino acid (AA) levels in different brain areas of WT and *lh/lh* mice has been conducted. In particular, we pointed out our attention to the levels of the main excitatory neurotransmitter, glutamate and the related non-neurotransmitter amino acid, glutamine (Table 1). Moreover, we measured the levels of the inhibitory neurotransmitter, GABA (Table 1). All these AAs had significantly higher concentration in the cortex of *lh/lh* mice brain, whereas they had significantly lower concentration in the cerebellum. Furthermore, glutamate content had significantly smaller concentration in the diencephalon. No other significant differences were observed between *lh/lh* and WT mice.

3.4. Amino acid levels after development of kindling in WT mice

After the development of kindling in WT mice, significant changes in AA levels were observed in comparison to basal conditions. Glutamate contents were found significantly increased in all brain areas studied with the exception of CB where concentration increased non-significantly and DE where a significant reduction was noticed. The levels of glutamine were found significantly increased in all brain areas (Table 2). GABA levels were found significantly decreased in all brain areas (Table 2).

Table 1
GABA, glutamate and glutamine levels in the different brain areas of WT and *lh/lh* mice in basal conditions

Amino acid	Brain area	WT mice	*Lh/lh* mice
GABA	Cortex	6.88±0.36	21.76±0.88**
	Hippocampus	4.86±0.33	5.57±0.63
	Diencephalon	9.46±1.05	8.13±0.73
	Brain stem	7.54±0.21	7.21±0.35
	Cerebellum	7.65±0.32	3.88±0.34**
Glutamate	Cortex	3.02±0.15	8.54±0.90**
	Hippocampus	3.53±0.59	2.53±0.35
	Diencephalon	5.62±0.41	3.58±0.49*
	Brain stem	1.73±0.29	2.40±0.24
	Cerebellum	6.63±0.78	2.90±0.33**
Glutamine	Cortex	2.03±0.20	7.39±0.50**
	Hippocampus	1.46±0.18	1.51±0.14
	Diencephalon	2.65±0.34	1.82±0.36
	Brain stem	1.84±0.21	1.66±0.13
	Cerebellum	1.94±0.19	0.77±0.09**

Data, expressed as μmoles/g wwt, are mean±S.E.M. of 10 different experiments.
* $P \leq 0.05$.
** $P \leq 0.01$, significantly different from WT mice.

Table 2
Percentage of modification of GABA, glutamate and glutamine levels in the different brain areas of WT and *lh/lh* mice after kindling

Amino acid	Brain area	WT mice	*Lh/lh* mice
GABA	Cortex	2.68±0.09 (−61%)**	7.67±0.68 (−64.7%)**
	Hippocampus	2.83±0.19 (−41.8%)**	5.92±0.42 (+6.3%)
	Diencephalon	5.94±0.55 (−37.2%)**	7.64±0.53 (−6%)
	Brain stem	2.91±0.25 (−61.4%)**	7.24±0.67 (+0.4%)
	Cerebellum	3.59±0.17 (−53.1%)**	5.37±0.32 (+38.4%)**
Glutamate	Cortex	6.14±0.29 (+103.3%)**	2.10±0.21 (−75.4%)**
	Hippocampus	7.44±0.7 (+110.8%)**	2.07±0.14 (−18.2%)
	Diencephalon	3.75±0.3 (−33.3%)*	0.93±0.17 (−74%)**
	Brain stem	4.42±0.65 (+155.5%)**	1.83±0.08 (−23.7%)
	Cerebellum	8.08±0.5 (+21.9%)	1.34±0.21 (−53.8%)**
Glutamine	Cortex	3.27±0.19 (+61.1%)**	2.54±0.34 (−65.6%)**
	Hippocampus	3.73±0.31 (+155.5%)**	2.4±0.13 (+64.9%)**
	Diencephalon	4.13±0.22 (+55.8%)**	1.10±0.11 (−39.6%)**
	Brain stem	3.47±0.24 (+88.6%)**	2.14±0.33 (+28.9%)
	Cerebellum	4.07±0.53 (+119.8%)**	1.56±0.28 (+102.6%)**

Data, expressed as percentage of modification in AA brain levels before and after kindling.
* $P \leq 0.05$.
** $P \leq 0.01$, significantly different from basal condition.

3.5. Amino acid levels after development of kindling in lh/lh mice

A statistical analysis for significant differences in amino acid levels after the development of kindling in *lh/lh* mice in comparison to basal conditions has been conducted. In particular, *lh/lh* mice showed a significant decrease of glutamate content in the CX, DE and CB (Table 2). The levels of glutamine were found significantly increased in the HI and the CB and decreased in the CX and DE. GABA levels were found significantly increased in the CB and decreased in the CX of kindled *lh/lh* mice (Table 2).

3.6. Comparison between the variations of amino acid levels after development of kindling in lh/lh and WT mice

As above reported, after the development of kindling, the contents of amino acids in the brain change significantly. Differences between the two strain responses to this treatment are evident. In particular, glutamate concentrations significantly decreased in the CX, DE and CB and did not significantly change in the HI and the BS of *lh/lh* mice,

whereas they are increased in the CX, the HI and the BS of WT mice. Glutamine contents were significantly increased in all brain areas of kindled WT mice and in contrast, significantly decreased in the CX and DE whereas significantly increased in the HI and CB of *lh/lh* mice. GABA levels were significantly reduced in all brain areas of WT mice, whereas they were significantly increased in the CB, decreased in the CX and unchanged in the other brain areas of *lh/lh* mice (Table 2).

4. Discussion

Both strains of mice used in the present study were tested after 60 days of age, when in mice's brains, the expression of most of the receptor subtypes is considered complete (Engstrom and Woodbury, 1988; Musumeci et al., 2000). Marked differences in AA levels were found between the two strains of mice in basal conditions. Alterations in the metabolism of several amino acids, especially glutamate, aspartate and GABA have been reported in various genetic models of epilepsy (Lasley, 1991; Lasley and Yan, 1994; Meldrum et al., 1999). It has to be underlined that all these three AAs play a role in the metabolism and therefore our measurements are most probably influenced by this factor. Microdialysis experiments would give a better idea of the real change in the neurotransmitters levels, however, this difficulty is affecting the comparison between data before and after kindling in the same strain but most probably not when comparing the amounts between the two strains. Lethargic (*lh/lh*) mice represent a genetic animal model of absence epilepsy, characterized by a defective β_4 subunit of voltage-gated Ca^{2+} channels. Further brain abnormalities reported for this strain of mice are: an increased $GABA_B$ receptor expression and binding in some brain areas (Hosford et al., 1999), a reduced excitatory synaptic transmission in thalamic neurones (Caddick et al., 1999), increases in peak current densities of low voltage-activated Ca^{2+} currents (Zhang et al., 2002), increase in GAD67 expression in nucleus reticularis thalami neurones (Lin et al., 1999b). *Lh/lh* mice generally develop spontaneous seizures and ataxia after the 3rd week of life and maintain them for the rest of their lifetime (Hosford and Wang, 1997). In the present work, we did not find any difference in the number and duration of slow wave discharges (SWDs) both after acute administration of PTZ or kindling (data not shown). Indicating that changes induced by kindling do not influence the thalamo-cortical circuitry involved in the generation of absence seizures in this animal model of epilepsy.

5. Pentylenetetrazole-induced seizures: acute administration and kindling

$GABA_A$ receptors are the most important inhibitory receptors of the central nervous system. They are composed of 5 subunits, each of which contains binding sites that can modulate the Chlorum-induced conductance. Numerous studies have shown that an insufficient synaptic inhibition by GABA on $GABA_A$ receptors may generate and/or contribute to the propagation of seizures (Corda et al., 1991; McNamara et al., 1989).

Up to date, data regarding a possible variation in the expression of the subunits of $GABA_A$ receptors in *lh/lh* mice are not present in literature. Pentylenetetrazole (PTZ) acts as an antagonist of GABA on the GABA–benzodiazepine–Cl^- ionophore receptor complex ($GABA_A$; for a review see Owens and Kriegstein, 2002). Repeated administration of PTZ subconvulsive doses (20–30 mg/kg) induces permanent changes in mouse brain, which lead to a kindling status that is usually persistent. In particular, the following changes have been reported: increased glutamate binding (da Silva et al., 1998), different expression in the subunit composition of some receptors (Ekonomou et al., 2001; Ekonomou and Angelatou, 1999), reduction of $GABA_A$ receptor density (Psarropoulou et al., 1994), increase in aspartate release from hippocampal neurones (Schroeder et al., 1999).

The CD_{50} values for PTZ-induced seizures were similar in *lh/lh* and WT mice, but the onset of kindled seizures was found significantly different. The different effects not produced by PTZ in this study suggest that in basal condition the GABA-benzodiazepine inhibitory inputs are unaltered by the mutation in voltage-activated Ca^{2+} channels, but mutation might promote the faster development of kindling in *lh/lh* mice than control.

It is not possible to exclude that the other abnormalities above-mentioned might also interfere during the acquisition period of the kindling state. Additional studies could be performed in *lh/lh* mice in order to better characterize whether the higher susceptibility to develop PTZ-induced kindling depends on similar mechanisms already described in other strains of mice (De Sarro et al., 2004a,b; Musumeci et al., 2000).

6. Amino acids concentration before and after kindling

Different amino acid concentrations in the brain might explain the different seizure susceptibility between various strains of mice (Leech and McIntyre, 1976; McNamara et al., 1989; Chapman et al., 1987; De Sarro et al., 2004a). Clear differences between WT and *lh/lh* mice were evident already in basal conditions. Our results show that all the AAs studied were significantly higher in the CX and lower in the CB of *lh/lh* mice compared to the WT mice and moreover, glutamate content was lower in the DE of *lh/lh* mouse. Since excitatory and inhibitory neurotransmitters are higher or lower in the same brain area, it seems impossible to correlate the different development of kindling in the two strains with an imbalance between the considered neurotransmitters levels, and it is then possible that this difference

is only due to the mutated Ca^{2+} channel β_4 subunit. We do not know if other brain abnormalities in *lh/lh* mice may induce a higher susceptibility to develop kindled seizures. It will be of interest to compare *lh/lh* mice with similar models lacking other Ca^{2+} channel subunits in order to assess the relative epileptic potential of such channel and to help the identification of potential targets for therapeutic interventions. Brain increase in $GABA_B$ receptor binding has been reported in *lh/lh* mice (Lin et al., 1993) but it is unclear whether this alteration contributes causally to the faster development of PTZ kindling. In addition, an increase of GAD67 mRNA in GABA neurons has been found in *lh/lh* mouse (Lin et al., 1999b); this has also been observed in particular conditions of increased neuronal activity: rat injected with kainate in the hippocampus (Feldblum et al., 1990); lesion of the inferior olive-climbing fiber projection (Litwak et al., 1990). It is therefore uncertain whether the increased GAD67 expression in *lh/lh* mice is the cause or the result of absence seizures and perhaps it might be involved in the faster development of kindling observed in this strain.

The development of chemical kindling induces permanent changes in mouse brain (Ekonomou et al., 2001; da Silva et al., 1998). The amino acid brain level modifications during seizure itself could result from both the kindling process and the physiological shock induced by the seizure. When the kindling status is reached, significant variations in AA levels are noticeable in the WT mice. These consist mainly in an increase in glutamate and glutamine content, except in the DE where glutamate concentration was decreased, and a decrease of GABA levels in all brain areas.

In contrast with the data regarding WT mice, in *lh/lh* mice, we did not find an increase in glutamate contents, otherwise a reduction in all brain areas, significative only in the CX, DE and CB. Glutamine content was found significantly decreased in the CX and DE, significantly increased in the HI and CB. The discrepancy in content of glutamate/glutamine observed in both strains might be due to compartmentation of neuronal glutamate metabolism in vesicular and cytosolic pools, as recently suggested by Eloquayli et al. (2003). The generally higher levels of glutamine in kindled mice compared to basal condition could be interpreted as an augmented rate of glutamate uptake and conversion to glutamine by glial cells with subsequent passive release (see Kaura et al., 1995). On the other hand, GABA content was significantly reduced only in the CX and significantly increased in the CB, whereas it remained stable in the other brain areas considered.

The possible involvement of glutamate as the endogenous excitatory trigger in seizure initiation and the involvement of glutamatergic neurotransmission system in the spread of epileptic seizure have been reviewed several times (Bradford and Peterson, 1987; Chapman et al., 1987; Kaura et al., 1995; De Sarro et al., 2004a).

The present data demonstrate that *lh/lh* mice reached the kindled status induced by PTZ differently than the WT mice and it is also different from other mice strains (De Sarro et al., 2004b). The opposite changes observed in *lh/lh* and WT mice are likely due to the differences in GABAergic inhibitory system and glutamatergic system previously reported between these mice's strains (Lin et al., 1999b; Caddick et al., 1999; Hosford et al., 1999). In conclusion, our findings indicate that *lh/lh* mice show a range of biochemical and behavioral changes, correlated in particular with a higher susceptibility to develop kindled seizures. However, it is difficult to draw conclusions regarding the neurotransmitter system principally involved in the higher susceptibility to develop kindling in *lh/lh* mice. We suppose that the role of the defective β_4 subunit of voltage Ca^{2+} channels deserves to be further investigated.

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