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Amino acid levels in some brain areas of inducible nitric oxide synthase knock out mouse (iNOS$^{-/-}$) before and after pentylenetetrazole kindling

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Abstract

Inducible nitric oxide synthase knock-out (iNOS$^{-/-}$) mice are valid models of investigation for the role of iNOS in patho-physiological conditions. There are no available data concerning neuroactive amino acid levels of iNOS$^{-/-}$ mice and their behaviour in response to pentylenetetrazole (PTZ). We found no significant differences in the convulsive dose 50 (CD_{50}) between iNOS$^{-/-}$ and control (iNOS$^{+/+}$) mice, however, iNOS$^{-/-}$ mice reach the kindled status more slowly than control, suggesting that in basal condition the GABA-benzodiazepine inhibitory inputs are unaltered by iNOS mutation. Clear differences between iNOS$^{+/+}$ and iNOS$^{-/-}$ mice amino acid concentrations were evident both in basal conditions and after kindling. Our results show that aspartate was significantly lower in all brain areas studied except the brain stem whereas glutamate and glutamine were significantly higher in the cortex, hippocampus and brain stem. GABA was slightly and not significantly higher in the cortex, hippocampus and brain stem, whereas taurine was significantly higher in all areas except diencephalon and glycine was significantly lower in the diencephalon and cerebellum. In this context, the inability of iNOS$^{-/-}$ mice to increase the NO levels following PTZ administrations indicate that NO might play a pro-epileptogenic role in the genesis and development of some types of epilepsy. Since there is no correlation between neurotransmitter levels and the development of kindling, it is possible to exclude that the difference between the two strains is due to an imbalance between the considered neurotransmitters, and it is then possible that this difference is due to the presence of iNOS, which might be involved in long term plasticity of the brain.

Keywords: Epilepsy; Pentylenetetrazole kindling; Neuroactive amino acids; Seizures; Inducible nitric oxide knock out mice

1. Introduction

Nitric oxide (NO) is one of the smallest, diffusible, biologically active messenger molecules. It is synthesized from the amino acid L-arginine by NO synthase (NOS), and appears to play a crucial role in many physiological and pathophysiological processes (Szabó, 1996; Rundfeldt et al., 1995; Moncada et al., 1991; Lowenstein et al., 1994). The NOS isoenzymes in the vascular endothelial cells (ecNOS) and in the neurons (bNOS) are present under physiological conditions, whereas the inducible NOS (iNOS) is expressed in response to external stimuli and produces high concentrations of NO (Szabó, 1996). Treatment of the seizure disorders might benefit a more thorough understanding of the role of NO in neuronal hyperexcitability and thus in epilepsy (Tutka et al., 2002). However, it is difficult to make a clear conclusion on the involvement of NO in epileptiform activity. A number of studies have shown that the modification of NO synthesis exerts contrasting effects upon animal convulsions, depending on the model of seizures and/or the dose, on pretreatment time or type of NO pathway modulators used (De Sarro et al., 1991, 1993; Haberny et al., 1992; Moncada et al., 1992; Rondouin et al., 1992, 1993; Tutka et al., 1996; Urbanska et al., 1996; Rundfeldt et al., 1995), and on brain structures and age of animals (Libri et al., 1997; de Vasconcelos et al., 2000).

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Rundfeldt and coworkers (1995) demonstrated in their study the duality of action of NO observing in their model that the threshold for seizures after cortical electrical stimulation in rats was increased by low doses of L-NOARG (1–10 mg/kg i.p.), whereas high doses (40 mg/kg, i.p.) lowered the threshold for seizures. However, these discrepancies might result from the actions of 7-nitroindazole, L-NAME and other NO synthase inhibitors that could be unrelated to brain NO synthase inhibition. For example, we have previously shown that anticonvulsant effects of 7-nitroindazole were related to increase the brain levels of dopamine and noradrenalin and that both anticonvulsant effects and changes in catecholamine content were antagonized by a pretreatment with α-methyl-paratyrosine, an agent inhibiting the synthesis of catecholamines (De Sarro et al., 2000) confirming data of other authors (Silva et al., 1995; Desvignes et al., 1999). These apparently divergent data do not, however, exclude that NO might play a different role in various models of epileptic disorders nor that it may either act as an anticonvulsant or proconvulsant agent, depending on the experimental procedures and/or particular brain structures as previously suggested by Libri et al. (1997).

Genetic animal models have contributed significantly to our understanding of the aetiopathologies of epilepsies (Buchhalter, 1993; Burgess and Noebels, 1999). The iNOS knock out mice ($iNOS^{-/-}$) have been widely used to determine the role of this isoenzyme for many physiological and pathophysiological conditions, they have been mainly used to analyze the involvement of iNOS in the gastrointestinal tract, cardiovascular system, inflammation and ischemia (Samdani et al., 1997; Mashimo and Goyal, 1999; Cuzzocrea et al., 2002). It is also widely accepted that NO is involved in many processes in the brain, and that in the Central Nervous System (CNS) it can be synthesized both by nNOS and iNOS (Heneka and Feinstein, 2001; Szabó, 1996). The latter is known to produce NO following stress conditions such as ischemia or seizures (Heneka and Feinstein, 2001). Up to date, there are no available data regarding the different role played by the two isoforms during seizures, the aim of this study is to add knowledge to role played by NO derived from iNOS in epilepsy. Furthermore, there are no available data concerning the actual concentrations of neurotransmitters in basal conditions in the mutant mice lacking of iNOS. In this study, we have evaluated the 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). We compared the convulsive dose 50 (CD_{50}) between $iNOS^{-/-}$ mice and their respective littermates control mice. 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

iNOS-Deficient Mice ($iNOS^{-/-}$; B6;129P2-/Nos2^tm1Lau //J) were originally obtained from The Jackson Laboratories (Bar Harbor, Maine, USA) and then colonies were maintained at the animal house of the Faculty of Pharmacy, University of Catanzaro (Catanzaro, Italy). As control animals, we have used the wild type littermates ($iNOS^{+/+}$) expressing the enzyme. 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 weighted 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 subcutaneously (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 (30×30×30 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 14 consecutive weeks in the morning between 9:00 and 11:00. In each experiment, the mice were placed in a Plexiglas box (30×30×30 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., 2000). The maximum response was recorded for each animal. The mice in each group were also observed for latency and duration of seizure response (De Sarro et al., 2000). 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 14 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 iNOS$^{-/-}$ and iNOS$^{+/+}$ mice at 60 days of age or 10 days after the development of kindling. Brain areas have been chosen within the areas primary involved in the generation and maintenance of PTZ-induced seizures (cortex, hippocampus, diencephalon, brain stem; Brevard et al., 2006; Oishi and Suenaga, 1982) and, as a control, we have used the cerebellum. Animals were decapitated without anaesthesia, 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 0.1 N 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 iNOS$^{-/-}$ and iNOS$^{+/+}$ mice was statistically compared using Fisher's exact probability test (data not shown in the 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 and mean total duration were 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 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 differences ($F=0.168$; $P=0.771$) in the CD_{50} values between iNOS$^{-/-}$ and iNOS$^{+/+}$ mice were observed. In particular, the CD_{50} values (±95% confidence limits) calculated following s.c. injection of PTZ were 59.13 (45.45–76.93) for iNOS$^{-/-}$ mice and 52.55 (41.89–65.90) for control mice. No significant differences ($P>0.05$) were observed in the mean latency to onset and mean total duration.

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

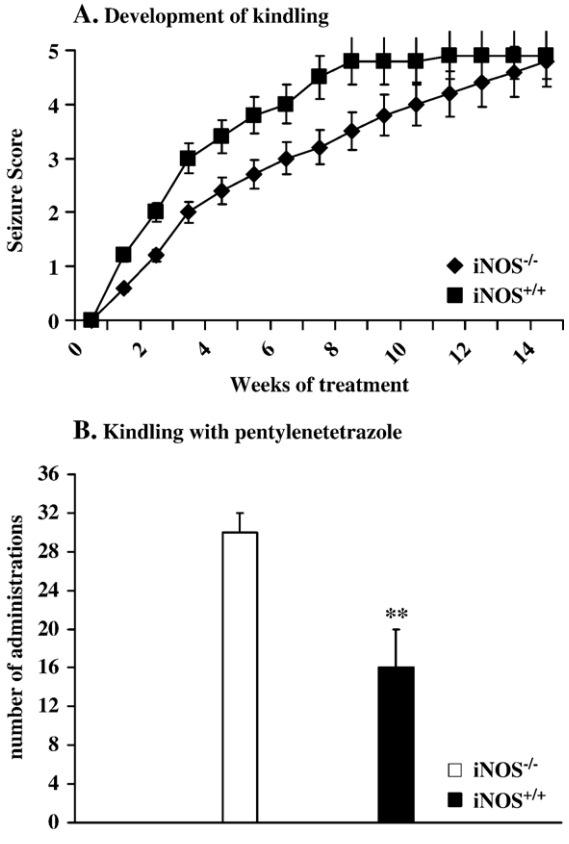

Fig. 1. Development of PTZ kindling. Effects of the repeated administration of PTZ on the manifestation of kindling during every other day treatment with PTZ (30 mg/kg, s.c.). (A) Ordinate shows the seizure score, abscissae show the weeks of repeated treatment. (B) Ordinate shows the number of administration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

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). iNOS$^{+/+}$ mice showed a more rapid development of kindling to PTZ during the 14 weeks of treatment than iNOS$^{-/-}$ mice, and consequently, the number of PTZ injections required to develop a phase 4 kindled convulsion was significantly different between the two strains ($P<0.05$).

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 14 weeks. In particular, none of the animals convulsed on the first administration and only 1/20 and 2/20, for iNOS$^{+/+}$ and iNOS$^{-/-}$ mice, respectively, did not reach the kindling status after 14 weeks. Analysis of variance of both latency to onset and mean total duration indicated that there was no significant difference ($P>0.05$) between the two different strain of mice.

3.3. Amino acid levels in basal conditions

A statistical comparison between the amino acid (AA) levels in different brain areas of iNOS$^{+/+}$ and iNOS$^{-/-}$ 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). Glutamate concentrations were significantly higher in the cortex ($P\leq0.001$), hippocampus ($P\leq0.05$) and brain stem ($P\leq0.001$) of iNOS$^{-/-}$ mice, whereas there were significantly lower concentration in the diencephalon ($P\leq0.001$). Aspartate content resulted significantly lower in all brain areas studied (for detailed P values see Table 1), except in the brain stem, of iNOS$^{-/-}$ mice than in iNOS$^{+/+}$. Furthermore, glutamine had significantly higher concentrations in the cortex ($P\leq0.05$), hippocampus ($P\leq0.005$), brain stem ($P\leq0.005$) and cerebellum ($P\leq0.005$) of iNOS$^{-/-}$ mice (Table 1).

Table 1
Aspartate, glutamate and glutamine levels in different brain areas of iNOS$^{+/+}$ and iNOS$^{-/-}$ mice in basal condition

Amino acid	Brain area	iNOS$^{+/+}$	iNOS$^{-/-}$
		$n=15$	$n=15$
Aspartate	Cortex	3.89±0.48	2.43±0.11 ***
	Hippocampus	3.42±0.12	2.16±0.08 ***
	Diencephalon	5.62±0.23	3.82±0.14 **
	Brain Stem	3.84±0.21	3.85±0.19
	Cerebellum	5.94±0.18	2.92±0.09 ***
Glutamate	Cortex	4.05±0.21	6.45±0.42 ***
	Hippocampus	4.21±0.33	5.32±0.21 *
	Diencephalon	5.39±0.37	2.88±0.19 ***
	Brain Stem	1.94±0.13	3.53±0.23 ***
	Cerebellum	5.61±0.24	4.91±0.42
Glutamine	Cortex	2.18±0.24	3.12±0.16 *
	Hippocampus	2.22±0.12	3.16±0.19 **
	Diencephalon	3.42±0.44	3.12±0.21
	Brain Stem	1.44±0.31	2.92±0.21 **
	Cerebellum	2.82±0.13	3.69±0.13 **

Data, expressed as μmol/g wwt, are mean±SEM.
* $P\leq0.05$; ** $P\leq0.005$; *** $P\leq0.001$, significantly different from iNOS$^{+/+}$.

Table 2
GABA, glycine, taurine levels in the different brain areas of iNOS$^{+/+}$ and iNOS$^{-/-}$ mice in basal condition

Amino acid	Brain area	iNOS$^{+/+}$	iNOS$^{-/-}$
		$n=15$	$n=15$
GABA	Cortex	6.91±0.28	7.13±0.35
	Hippocampus	5.37±0.17	5.39±0.34
	Diencephalon	8.74±0.45	8.41±0.63
	Brain Stem	7.25±0.31	7.79±0.44
	Cerebellum	6.35±0.45	6.73±0.39
Glycine	Cortex	3.03±0.47	2.74±0.19
	Hippocampus	1.98±0.32	2.38±0.10
	Diencephalon	4.35±0.18	3.87±0.17 *
	Brain Stem	4.98±0.41	4.37±0.32
	Cerebellum	3.06±0.31	2.94±0.16
Taurine	Cortex	5.88±0.34	8.35±0.44 ***
	Hippocampus	5.43±0.25	7.57±0.22 ***
	Diencephalon	4.93±0.44	4.51±0.37
	Brain Stem	3.65±0.17	4.39±0.35 *
	Cerebellum	6.14±0.11	6.84±0.32 *

Data, expressed as μmol/g wwt, are mean±SEM.
* $P\leq0.05$; ** $P\leq0.005$; *** $P\leq0.001$ significantly different from iNOS$^{+/+}$.

Moreover, we measured the levels of the inhibitory neurotransmitters, GABA, glycine and taurine (Table 2). No significant differences were observed between iNOS$^{-/-}$ and iNOS$^{+/+}$ mice in GABA brain contents. Glycine brain content was not significantly modified in iNOS$^{-/-}$ mice with the exception of diencephalon where a significant lower level ($P\leq0.05$) was measured in iNOS$^{-/-}$ mice. Instead, taurine content were significantly higher in the cortex ($P\leq0.001$), hippocampus ($P\leq0.001$), brain stem ($P\leq0.05$) and cerebellum ($P\leq0.05$) whereas a no significant lower level was observed in the diencephalon of iNOS$^{-/-}$ mice (Table 2).

3.4. Amino acid levels after kindling development in iNOS$^{+/+}$ mice

After the development of kindling in iNOS$^{+/+}$ 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 diencephalon where a no significant reduction was noticed (for detailed P values see Table 3). The levels of glutamine were found significantly increased in all brain areas (for detailed P values see Table 3). Aspartate contents were found significantly decreased in the cortex ($P\leq0.005$), diencephalon ($P\leq0.05$) and cerebellum ($P\leq0.001$) and increased in the brain stem ($P\leq0.05$; Table 3). GABA levels were found significantly decreased in all brain areas (for detailed P values see Table 4). Glycine contents were found significantly decreased in the cortex ($P\leq0.05$), diencephalon ($P\leq0.005$) and cerebellum ($P\leq0.05$), whereas taurine levels significantly increased in the hippocampus ($P\leq0.001$) and cerebellum ($P\leq0.005$; Table 4).

Table 3
Aspartate, glutamate and glutamine levels in the different brain areas of iNOS$^{+/+}$ and iNOS$^{-/-}$ mice after kindling and percentage of modification versus basal conditions

Amino acid	Brain area	iNOS$^{+/+}$	iNOS$^{-/-}$
		$n=15$	$n=13$
Aspartate	Cortex	2.67±0.24 (−31.4%) **	4.81±0.28 (+97.7%) ***
	Hippocampus	2.69±0.42 (−21.2%)	4.17±0.31 (+93.1%) ***
	Diencephalon	4.59±0.52 (−18.4%) *	4.92±0.27 (+28.8%) **
	Brain stem	5.82±0.37 (+51.7%) *	4.89±0.25 (+27%) *
	Cerebellum	3.66±0.21 (−38.4%) ***	4.62±0.37 (+58.2%) ***
Glutamate	Cortex	8.59±0.42 (+112.1%) ***	7.77±0.37 (+20.5%) *
	Hippocampus	8.77±0.63 (+108.4%) ***	6.84±0.52 (+28.6%) *
	Diencephalon	3.91±0.36 (−27.4%)	3.92±0.23 (+36.1%) *
	Brain stem	4.40±0.32 (+126.9%) ***	3.85±0.31 (+9.1%)
	Cerebellum	7.21±0.71 (+28.5%) *	7.56±0.35 (+53.9%) ***
Glutamine	Cortex	3.24±0.11 (+48.4%) ***	4.35±0.22 (+39.4%) ***
	Hippocampus	5.16±0.44 (+132.4%) ***	4.24±0.27 (+34.2%) **
	Diencephalon	5.01±0.16 (+46.6%) *	2.96±0.18 (−5.1%)
	Brain Stem	2.81±0.19 (+95.3%) ***	2.54±0.21 (−13.1%) *
	Cerebellum	4.93±0.33 (+74.8%) ***	3.47±0.25 (−5.9%)

Data, expressed as μmol/g wwt, are mean±SEM.
* $P\leq 0.05$; ** $P\leq 0.005$; *** $P\leq 0.001$ significantly different from basal condition.

3.5. Amino acid levels after kindling development in iNOS$^{-/-}$ mice

The statistical analysis for significant differences in amino acid levels after the development of kindling in iNOS$^{-/-}$ mice in comparison to basal conditions showed a significant increase of glutamate contents in the cortex ($P\leq 0.05$), hippocampus ($P\leq 0.05$), diencephalon ($P\leq 0.05$) and cerebellum ($P\leq 0.001$). A significant increase of glutamine content was evidenced in the cortex ($P\leq 0.001$) and hippocampus ($P\leq 0.005$), whereas a significant decrease was observed in the brain stem ($P\leq 0.05$; Table 3). The levels of aspartate were found significantly increased in all brain areas considered (for detailed P values see Table 3). GABA concentrations were found significantly increased in the cortex ($P\leq 0.05$) and decreased in the brain stem ($P\leq 0.001$) of kindled iNOS$^{-/-}$ mice. Glycine contents vwere found significantly increased in all the brain areas studied (for detailed P values see Table 4), whereas taurine levels significantly increased in the cortex ($P\leq 0.05$) and hippocampus ($P\leq 0.005$) and decreased in the brain stem ($P\leq 0.05$) only (Table 4).

3.6. Comparison between the variations of amino acid levels after kindling development in iNOS$^{-/-}$ and iNOS$^{+/+}$ 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 were found more markedly increased in iNOS$^{+/+}$ than in iNOS$^{-/-}$ mice in

Table 4
GABA, glycine and taurine levels in the different brain areas of iNOS$^{+/+}$ and iNOS$^{-/-}$ mice after kindling, and percentage of modification versus basal conditions

Amino acid	Brain area	iNOS$^{+/+}$	iNOS$^{-/-}$
		$n=15$	$n=13$
GABA	Cortex	2.85±0.17 (−58.8%) ***	8.61±0.45 (+20.7%) *
	Hippocampus	2.81±0.09 (−47.6%) ***	5.65±0.28 (+4.8%)
	Diencephalon	6.06±0.21 (−30.6%) *	8.88±0.48 (+5.6%)
	Brain Stem	1.78±0.28 (−75.2%) ***	3.92±0.23 (−49.7%) ***
	Cerebellum	2.46±0.19 (−61.2%) ***	6.59±0.24 (−2.1%)
Glicine	Cortex	2.04±0.16 (−32.6%) *	4.05±0.19 (+47.8%) ***
	Hippocampus	1.77±0.32 (−10.4%)	3.28±0.21 (+37.8%) **
	Diencephalon	2.71±0.21 (−37.7%) **	4.68±0.24 (+20.9%) *
	Brain Stem	5.57±0.36 (+12.0%)	6.35±0.35 (+45.3%) **
	Cerebellum	2.05±0.24 (−32.9%) *	3.45±0.16 (+17.6%) *
Taurine	Cortex	6.61±0.37 (+12.4%)	10.32±0.58 (+23.6%) *
	Hippocampus	8.41±0.22 (+54.9%) ***	10.28±0.44 (+35.8%) **
	Diencephalon	5.19±0.18 (+5.2%)	4.65±0.24 (+3.1%)
	Brain Stem	3.02±0.45 (−17.2%)	3.17±0.19 (−27.8%) *
	Cerebellum	7.69±0.34 (+25.4%) **	7.79±0.45 (+13.9%)

Data, expressed as μmol/g wwt, are mean±SEM.
* $P\leq 0.05$; ** $P\leq 0.005$; *** $P\leq 0.001$, significantly different from basal condition.

the cortex, hippocampus and brain stem. Furthermore, kindling treatment induced opposite effects in diencephalon glutamate levels of the two strains of mice. In particular, a significant decrease in iNOS$^{+/+}$ and a significant increase in iNOS$^{-/-}$ mice was seen. Glutamine contents were significantly increased in all brain areas of kindled iNOS$^{+/+}$ mice, whereas the glutamine increase was found less marked and limited to the cortex and hippocampus of iNOS$^{-/-}$ mice; glutamine content was significantly decreased in the brain stem of this latter strain (Table 3). Aspartate levels significantly increased in all brain areas of iNOS$^{-/-}$ mice. In iNOS$^{+/+}$ mice a significant increase was observed only in brain stem, whereas in the cortex and cerebellum significant decreases were evidenced (Table 3).

GABA levels were significantly reduced in all brain areas of kindled iNOS$^{+/+}$ mice, whereas they were significantly increased in the cortex and significantly reduced in the brain stem of kindled iNOS$^{-/-}$ mice. Glycine concentrations were reduced in all brain areas of kindled iNOS$^{+/+}$ mice, even if significant changes were observed in the cortex, diencephalon and cerebellum only. Kindling treatment induced opposite effects in iNOS$^{-/-}$ mice, where glycine levels were found significantly increased. Taurine contents were significantly increased in the hippocampus and cerebellum of kindled iNOS$^{+/+}$ mice whereas they were found increased in the cortex and hippocampus and decreased in brain stem of kindled iNOS$^{-/-}$ mice (Table 4).

4. Discussion

Under physiological conditions, NO plays an important role in the modulation of a variety of central nervous system functions. The most important stimulus for the activation of nNOS is the opening of glutamate NMDA receptors and to some extent activation of AMPA/Kainate receptors (Bredt and Snyder, 1989, 1994). In turn, NO is also responsible for oxidizing some redox sites within the NMDA receptor complex and therefore, inhibits the necessary influx of Ca^{2+} for its activation acting as a negative feedback system (Lipton et al., 1994). Furthermore, a localized increase in neuronal activity has been associated to a NO-dependent increase in regional blood flow (Szabó, 1996). This raised the possibility that overactivation of NMDA receptors contributes to the pathogenesis of enhanced blood flow during epileptic seizures. However, the evidence for the role of NO in this disease remains controversial.

In early studies, it was found that L-arginine increased the severity of seizures following a subconvulsive dose of NMDA suggesting a proconvulsive role for NO (De Sarro et al., 1991, 1993; Mollace et al., 1991). In contrast, inhibition of NOS prolongs bicuculline-induced seizures (Theard et al., 1995). Direct administration of NO (330–800 μmol) into the rat brain has been attempted resulting in brief tonic convulsive episodes (Smith et al., 1991) and 3-morpholiNOSydnonimine (SIN-1), a NO donor, is convulsant in DBA/2 mice and genetically epilepsy-prone rats (Smith et al., 1996). Rundfeldt and co-workers (1995) demonstrated that NO behaves either as a convulsant or an anticonvulsant in the same animal model depending on the dose administered.

These apparently divergent data do not, however, exclude that NO might play a different role in various models of epileptic disorders nor that it may either act as an anticonvulsant or proconvulsant agent, depending on the experimental procedures and/or particular brain structures as suggested by Libri et al. (1997).

Both strains of mice used in the present study were tested after 60 days of age, when in mice 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; De Luca et al., 2005). It has to be underlined that the excitatory AAs play a role in the metabolism and therefore our measurements are most probably influenced by metabolic changes. Microdialysis experiments would give a better idea of the real change in the neurotransmitter levels; however, metabolism might affect the comparison between data before and after kindling in the same strain but most probably it does not affect comparison between the two strains.

iNOS$^{-/-}$ mice are considered a valid model to investigate the role of iNOS in physiological and pathological conditions. In the present work, we did not find any difference in the electrocortical pattern and in the number and duration of epileptic discharges both after acute administration of PTZ or kindling (data not shown). Indicating that changes induced by kindling do not influence the neuronal circuitry involved in the generation of kindled seizures in these animal models.

$GABA_A$ receptors are the most important inhibitory receptors of the central nervous system. 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 $GABA_A$ receptors subunits in iNOS$^{-/-}$ 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 (20–50 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 iNOS$^{-/-}$ and iNOS$^{+/+}$ mice, but the onset of kindled seizures was found significantly different, suggesting that in basal condition the GABA-benzodiazepine inhibitory inputs are unaltered by the mutation in iNOS, but this mutation might be responsible for the slower development of kindling in iNOS$^{-/-}$

mice than iNOS$^{+/+}$. Additional studies could be performed in iNOS$^{-/-}$ mice in order to better characterize whether the lower susceptibility to develop PTZ-induced kindling depends on similar mechanisms already described in other strains of mice (De Sarro et al., 2004a,b; De Luca et al., 2005; Musumeci et al., 2000).

Different brain concentrations of neuroactive amino acids might explain the different seizure susceptibility between various strains of mice (Leech and McIntyre, 1976; McNamara et al., 1989; De Sarro et al., 2004a; De Luca et al., 2005). Clear differences between iNOS$^{+/+}$ and iNOS$^{-/-}$ mice were already evident in basal conditions, and even if differently, amino acid concentrations were completely modified also after the development of kindling. Our results show that aspartate content was significantly lower in all the brain areas studied with the exception of brain stem whereas glutamate and glutamine levels were significantly higher in the cortex, hippocampus and brain stem of iNOS$^{-/-}$ mice compared to the iNOS$^{+/+}$ mice. In addition, GABA contents were only slightly and not significantly higher in the cortex, hippocampus and brain stem of iNOS$^{-/-}$ mice compared to the iNOS$^{+/+}$ mice, whereas taurine levels were significantly higher in all areas except diencephalon and glycine level was significantly lower in the diencephalon and cerebellum.

From these results, it would be expected that iNOS$^{-/-}$ mice would develop the kindling status faster than control mice, instead our observations indicate that the lack of this enzyme protects from the effects of repeated PTZ stimulation and therefore delays the development of kindling. This is in agreement with other reports indicating that NO has a proconvulsant action, and plays a potential role in mechanisms regulating seizure induction and propagation (Osonoe et al., 1994; Mülsch et al., 1994; Hara et al., 1996; Han et al., 2000, Maggio et al., 1995), furthermore, in a recent paper, it has been demonstrated that PTZ kindling in mice increases NOS activity (El-Abhar and El Gawad, 2003).

Since there is no direct/apparent correlation between neurotransmitter levels and the development of kindling, it is possible to exclude that the difference between the two strains of mice is due to an imbalance between the considered neurotransmitter levels before kindling, and it is then possible that this difference is only due to the presence of iNOS, which might be involved in long term plasticity of the brain. However, it has to be underlined that after kindling iNOS$^{-/-}$ mice in contrast to control mice present lower levels of glutamate and higher levels of GABA, therefore, it might be possible that the expression of iNOS regulates somehow synaptic activity facilitating the increase in glutamate content and reducing GABA levels, it is known that NO is able to modulate the presynaptic release of excitatory amino acids (Theard et al., 1995).

We do not know if other brain abnormalities in iNOS$^{-/-}$ mice may induce a lower susceptibility to develop kindled seizures. It will be of interest to compare iNOS$^{-/-}$ mice with similar models lacking other NOS enzymes in order to assess the relative epileptic potential of every single NOS isoform and to help the identification of potential targets for therapeutic interventions.

The development of chemical kindling induces permanent changes in mouse brain (Ekonomou et al., 2001; da Silva et al., 1998; De Luca et al., 2005). The amino acid level modifications in brain 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 iNOS$^{+/+}$ mice. These consist mainly in an increase in glutamate and glutamine content and a decrease of GABA levels in all brain areas. In contrast with the data regarding iNOS$^{+/+}$ mice, in iNOS$^{-/-}$ mice, we did not find a marked increase in glutamate contents, instead, a marked increase in aspartate levels was observed in all brain areas. Glutamine content was found significantly decreased in the brain stem and significantly increased in the hippocampus and the cortex of iNOS$^{-/-}$ mice in comparison to the iNOS$^{+/+}$ mice. 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 brain stem and significantly increased in the cortex, whereas it remained stable in the other brain areas considered, in contrast with the reduction observed in all brain areas of control mice. 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; Kaura et al., 1995; De Sarro et al., 2004a).

Glycine content was significantly increased in all brain areas and taurine level changes were less marked in the hippocampus and cerebellum of iNOS$^{-/-}$ mice, whereas it remained stable in the brain stem.

The present data demonstrate that iNOS$^{-/-}$ mice reach the kindled status induced by PTZ more slowly than iNOS$^{+/+}$ mice and it is also different from other mice strains (De Sarro et al., 2004b; De Luca et al., 2005). However, it is difficult to draw conclusions regarding the neurotransmitter system principally involved in the lower susceptibility to develop kindling in iNOS$^{-/-}$ mice. We suppose that the role of the deficit iNOS in epilepsy deserves to be further investigated. In this context, the inability of iNOS$^{-/-}$ mice to increase the NO levels following repeated administration of PTZ indicate that NO might play a pro-epileptogenic role in the genesis and development of some types of epilepsy. This is in agreement with other reports indicating a proconvulsive role for NO in the development of various type of kindling (al-Ghoul et al., 1995; Han et al., 2000; Park et al., 2001).

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