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GLUTAMATERGIC SYSTEM: ANOTHER TARGET FOR THE ACTION OF PORPHYRINOGENIC AGENTS

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Abstract – The N-methyl-diethyl-aspartate (NMDA) receptor has been reported to play an important role in several acute and chronic neuropathologic syndromes. 5-aminolevulinic acid (ALA) accumulates in acute porphyrias due to a deficiency in the heme biosynthetic pathway. Considering that glutamate uptake inhibition caused by ALA could be one of the reasons conducing to porphyric neuropathy, it was of interest to evaluate the effect of porphyrinogenic agents on NMDA glutamatergic system. To this end receptor levels and apparent affinity (Kd) were analyzed in mice brain cortex and cerebellum. NMDA levels were diminished after chronic Isoflurane anaesthesia in brain cortex. In cerebellum, a diminution was observed after acute Enflurane and Isoflurane and allylisopropylacetamide, while ethanol administration showed a significant increase. ALA administration diminished NMDA levels only in cerebellum. Affinity constant was only reduced in brain cortex after chronic Isoflurane treatment. In conclusion, glutamatergic system appears to be involved in the action of some of the porphyrinogenic drugs studied mainly in cerebellum. Receptors regulation should therefore be considered an important mechanism in the cellular response to specific drugs, with the aim of designing new therapies and elucidating the mechanisms leading to porphyric neuropathy and acute attack triggering.

Key words: Glutamatergic system, N-methyl-diethyl-aspartate, Porphyrinogenic agents, Porphyric neuropathy

INTRODUCTION

Glutamate is the most ubiquitous of the fast excitatory neurotransmitters in the brain (22). The synaptic actions of glutamate are mediated by two main types of receptors of the ionotropic and metabotropic classes (16).

Ionotropic glutamate receptors are widely distributed throughout the central nervous system (CNS), including the cerebral cortex, limbic regions and basal ganglia (16, 27, 35).

Abreviations: AIA, Allylisopropylacetamide**; Gris,** Griseofulvin; **ALA,** 5-aminolevulinic acid; **NMDA,** N-methyl-diethyl-aspartate; **CNS,** central nervous system; **PMSF,** phenylmethylsulfonyl fluoride

NMDA (N-methyl-diethyl-aspartate) receptor has been reported to play an important role in neuronal development, normal synaptic transmission, learning and memory, and in various acute and chronic neuropathologic syndromes (8, 23, 26). Uncontrolled activity leads to neuronal cell death contributing to the pathogenesis of various neural diseases and pathologic states. Therefore, the investigation of the function and the complex regulation of ionotropic glutamate receptors are crucial for the better understanding of basic brain functions such as learning and memory, as well as for the rational treatment of CNS diseases.

The heme precursor 5-aminolevulinic acid (ALA) accumulates in acute porphyrias due to a deficiency in the heme biosynthetic pathway (19). Although the biochemical support for the neurological manifestations of these disorders has

not been completely understood, it has been proposed that ALA accumulation could contribute to some of these manifestations (19, 10, 31). Acute intracerebral administration of ALA to rats induces body asymmetry, convulsions and death (12, 30, 36) that were prevented by glutamate receptor antagonists, suggesting that the activation of glutamatergic receptors was involved in the above indicated ALA effects (12).

Moreover, ALA has been reported to inhibit glutamate uptake and stimulate the release of this excitatory neurotransmitter from rat synaptosomes (2, 24).

Taking into account that glutamate uptake inhibition caused by ALA could be one of the reasons conducing to porphyric neuropathy, that ALA crosses the hematoencephalic barrier and as a consecuence of porphyrinogenic drugs a secondary increase of ALA is produced, it results of interest to evaluate the effect of porphyrinogenic agents on NMDA glutamatergic system. For this purpose receptor levels and apparent affinity (Kd) were analyzed in brain cortex and cerebellum of mice receiving anaesthetics Enflurane and Isoflurane, allylisopropylacetamide (AIA), veronal, ethanol and Griseofulvin (Gris) administered in doses and timing causing alterations on liver heme metabolism according to previous work (1, 3-6, 28). Results were compared with those obtained in ALA treated mice.

MATERIALS AND METHODS

Chemicals

Enflurane and Isoflurane were from Abbott Laboratories S.A. All other chemicals used were reagent grade obtained from Sigma Chem. Co., St. Louis, USA.

Animals

Albino male adult *CF1* mice (6-8 animals/group) weighing 25-30 g (6 weeks old) were maintained in controlled conditions and allowed free access to food (Purina 3, Asociación de Cooperativas Argentinas, San Nicolás, Buenos Aires, Argentine) and water. Animals received human care and were treated in accordance with the guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC). Animals were sacrificed at the same time of the day.

Treatments:

Treatment conditions were as follows, as described in our previous work (20).

- *Anaesthetics*: During the acute treatment animals received a single dose of 2 ml/kg (i.p.) of Enflurane or Isoflurane and were sacrificed 20 minutes after the injection. During the chronic treatment animals received 10 doses of 1 ml/kg (i.p., every 48 hours) and were sacrificed 20 minutes after the last injection.
- *AIA*: Animals received a single dose of 350 mg/kg (i.p.) (in ethanol:NaCl 0.9%; 1:3 v/v) 16 hours prior to sacrifice.
- *Veronal:* Animals were given one daily dose of veronal of 167 mg/kg (s.c.) during 3 days, and they were sacrificed 24 hours after the last dose.
- *Ethanol*: Animals received ethanol (30%, v/v) in the drinking water during a week.
- *Gris:* Gris was administered in two different ways: topical and oral: Topical Gris (50 mg/ml in corn oil) was applied on the back of each animal, 24 hours prior to sacrifice. In the diet, animals received 2.5% of Gris during 15 days.
- *Starvation:* Animals were deprived of food 24 hours prior to sacrifice.
- *ALA*: Acute treatment: Animals received a single dose of 40 mg/kg (i.p.) and were sacrificed 24 hours after injection. Chronic treatment: one dose of 40 mg/kg (i.p.), every 48 hours, during 15 days and were sacrificed 24 hours after the last injection.

Control animals received the vehicle or were exposed to the same experimental conditions and they were sacrificed at the same times as indicated for each particular treatment.

Homogenate preparation

Brain cortex and cerebellum were homogenized in 20 mM Tris-HCl buffer, pH 7.4 (1:20 w/v) containing 0.32 M sucrose, 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged for 10 min at 1,000xg and the supernatant was again centrifuged at 13,000xg for 30 min. The precipitate obtained was resuspended in 5 mM Tris-HCl buffer, pH 8.1 containing 50 μM $CaCl_2$ and centrifuged at 20,200xg for 20 min. The pellet was resuspended in the same homogenization solution and it was used to quantify NMDA.

NMDA receptor levels

NMDA levels were measured by the competitive ligand assay with [^{3}H]MK-801 according to Wong *et al.* (43) procedure slightly modified.

The incubation system contained in a final volume of 0.5 ml: 5 mM Tris-acetate buffer pH 7.5, 50 μM glutamate, 50 μM glycine, 30 μg of tissue extract and a saturating concentration of [^{3}H]MK-801 (80 nM) (AE 17.1 Ci/mmol). Unspecific union was carried out in presence of 100 μM of nonradioactive MK-801. The system was incubated for 3 hours at 28ºC. Incubation was finished by rapid filtration through Whatman GF/B filters, which were washed immediately with two 5-ml portions of ice-cold assay buffer. After filtration, filters were dried at 40ºC during 12 hours. Radioactivity on the filters was determined by liquid scintillation counting in standard vials of 10 ml of 18 mM 2.5-difeniloxazol (PPO) and 0.27 mM 2.2-p-fenilen-bis(5-feniloxazol) (POPOP) in toluene. Receptor NMDA levels were expressed as pmoles per mg protein. Scatchard analysis was performed to evaluate the effect on affinity constant (Kd) using different concentrations of [^{3}H]MK-801 ranging from 4 to 80 nM.

Protein concentration was estimated by the procedure of Lowry *et al.* (21) with a modification for measuring insoluble proteins.

Statistical analysis

Data were expressed as mean values ± s.d. Differences in mean values between treated and control

groups were evaluated using the analysis of variance (ANOVA) and $p<0.05$ was considered statistically significant.

RESULTS

Effect of porphyrinogenic agents on NMDA receptor levels

The effect of porphyrinogenic agents administration on NMDA receptor levels of brain cortex are shown in Figure 1. NMDA levels were diminished 40% ($p<0.05$) after chronic Isoflurane anaesthesia while no variations were observed after only one dose of this anaesthetic or after Enflurane. No changes were detected after other agents studied.

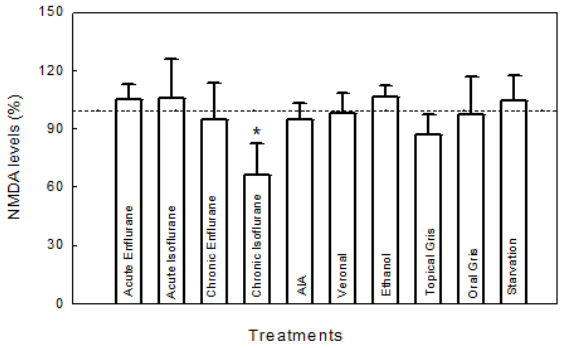

Figure 1. NMDA levels in brain cortex. Data represents mean value ± s.d. of each determination performed in triplicate and are expressed as percentage of each control. *p<0.05 significance of differences between treated and control groups. A unique control value (…) is given, because no significant differences were obtained in any of the controls after the administration of the corresponding vehicles. Experimental details are described in the text.

The effect of porphyrinogenic agents administration on cerebellum NMDA receptor levels are shown in Figure 2.

NMDA receptor levels were 50% ($p<0.05$) diminished in animals receiving acute Enflurane and Isoflurane, without significant alterations after chronic anaesthesia. Moreover AIA diminished 30% ($p<0.05$) receptor levels. Instead ethanol administration strikingly increased 50% ($p<0.05$) NMDA levels. No alterations were observed after veronal, Gris or starvation.

Effect of ALA on NMDA receptor levels

The effect of acute and chronic ALA administration on NMDA receptor levels in brain cortex and cerebellum are shown in Figure 3.

In cerebellum, NMDA receptor levels were 32% ($p<0.05$) diminished after acute and chronic ALA administration. However no significant changes were observed in brain cortex.

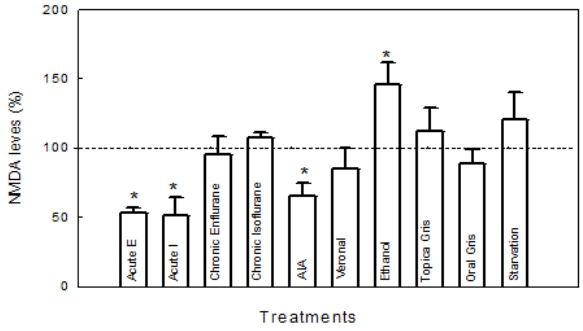

Figure 2. NMDA levels in cerebellum. E: Enflurane, I: Isoflurane. Data represents mean value ± s.d. of each determination performed in triplicate and are expressed as percentage of each control. *p<0.05 significance of differences between treated and control groups. Experimental details are described in legend to Fig. 1 and in the text.

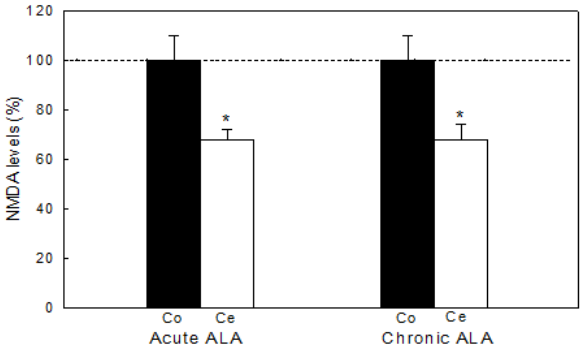

Figure 3. NMDA levels in brain cortex (Co) and cerebellum (Ce) after ALA treatment.
Data represents mean value ± s.d. of each determination performed in triplicate and are expressed as percentage of each control. *p<0.05 significance of differences between treated and control groups. Experimental details are described in legend to Fig.1 and in the text

Effect of porphyrinogenic agents and ALA on apparent affinity (Kd)

Scatchard plots of [^{3}H]MK-801 binding of chronic Isoflurane treated mice are shown in Figure 4. In cortex, chronic Isoflurane scatchard analysis revealed a 38% diminution of Kd .

No modification of this parameter was observed by effect of the other agents studied (data not shown).

DISCUSSION

Taking into account the generalized distribution of glutamate receptors in CNS, it is probable that its receptors were target of several therapeutic interventions.

It has been proposed a relation between glutamate receptors or glutamatergic transmition and chronic neurodegenerative diseases and in

squizofrenia (7, 25). At the moment on biochemical basis it has been proposed that ALA accumulation could contribute to some of these neurological manifestations (10, 19, 31). Emanuelli *et al.* (12) have demonstrated that induced convulsions by ALA could be prevented by glutamate receptors antagonists, suggesting that the activation of the glutamate receptor could be involved in ALA effects, these authors (13) also described that ALA inhibits glutamate uptake in astrocyte cultures, probably due to the oxidative damage provoked by this molecule. Villayandre *et al*. (42) demonstrated that treatment with chronic ALA *in vivo* produces changes in NMDA receptor levels. Enhancement of oxidative stress due to the ALA hypothesis, could be a probable mechanism explaining a diminution in ligand union values observed after ALA chronic treatment. We have actually found a reduction of NMDA levels in cerebellum, after one or several doses of ALA, without any modification of Kd.

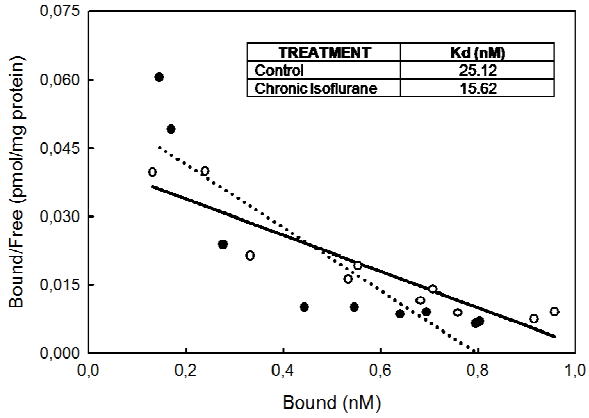

Figure 4. Scatchard plot of [^{3}H]MK-801 binding of NMDA receptors on the brain cortex of mice treated with chronic Isoflurane.
◯Control, ● Chronic Isoflurane. Results of the saturation binding assay using [^{3}H]MK-801 ranging from 4 to 80 nM were transformed to Scatchard plots. Experimental details are described in the text.

To compare whether porphyrinogenic agents does modify NMDA receptor in a similar way than ALA, we measured NMDA levels in animals receiving anaestethics and other xenobiotics.

Our results would indicate that agents studied affect glutamatergic system mainly in cerebellum and the major changes were detected on NMDA density. Although, in cortex chronic Isoflurane affected both parameters, affinity and density.

Glutamate receptor function is implicated in the pathophysiology of alcoholism (11, 40). Chronic ethanol treatment increases NMDA receptor binding (14, 15, 17, 38, 41), increases NMDA subunit expression (18, 39, 41) and potentiates a number of NMDA receptor-mediated responses including neurotoxic effects (9, 40). We have found an enhancement in NMDA levels in cerebellum after ethanol treatment.

Anaesthetics decreased NMDA levels depending on the length of the treatment. Ranft *et al*. (32) demonstrated *in vitro* that Isoflurane in amygdale culture, reduced intensity in synaptic signal followed NMDA receptor activation.

AIA diminished NMDA levels in cerebellum.

Deficits in NMDA-receptor-mediated glutamatergic neurotransmission have been described as biochemical mechanisms of age-associated deficits in cognitive functions (29). Activation of NMDA receptors has been reported to cause the formation of nitric oxide, a free radical gas, through nitric oxide synthase activation, which directly relates NMDA receptors to oxidative stress (33, 37).

So that, glutamatergic system appear to be involved in mediating the action of some of the porphyrinogenic drugs studied.

Rodriguez *et al.* (34) observed alterations in acetylcholine muscarine receptor (mAChR) mainly in cerebellum and hippocampus after administration of some porphyrinogenic drugs like Enflurane, Gris and ethanol. Although, ALA administration produced no alterations on mAChR, and cholinesterases activity.

Receptors regulation should therefore be considered an important mechanism explaining the cellular response to specific drugs, with the aim of designing new therapies and elucidating mechanisms leading to the porphyric neuropathy and the acute attack triggering.

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