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1. Methylmercury

Metals are strongly bound to many facets of modern human existence and, while some are biologically essential (copper, zinc, manganese, etc), others are extremely poisonous (as mercury, lead and cadmium). Mercury is a heavy metal that is widely distributed in the earth's crust. Both natural and anthropogenic sources contribute to the global cycling of this element (IPCS, 1989, ATSDR, 1994). In aquatic environments, inorganic mercury is converted to methylmercury (MeHg) by methanogenic bacteria present in sediments of fresh and oceanic water. MeHg is then bioaccumulated and bioconcentrated as it passes up the aquatic food chain. All fishes contain some MeHg and vertebrates at the top of the food chain contain the largest quantities.

This compound produces profound alterations on the developing central nervous system (CNS), and in adults it can lead to severe and permanent damage to the CNS (Clarkson, 1997). Although the latest reports about groups of people whose diet is based on fish and are exposed to MeHg do not confirm previous assessments of CNS damage (Myers et al., 2003), epidemic poisonings have occurred in Japan and Irak, resulting from the consumption of MeHg-contaminated fish and alkilmercury-treated seed grain, respectively. Neurological disorders in adults most frequently included constriction of the visual field with reduced visual acuity, paresthesia of the extremities and perioral region, impaired two-point discrimination in the extremities, impaired vibration and joint point sense, deafness to high tones, and ataxia. Neuropathology in these cases revealed characteristic

lesions of the cerebellum and cerebral cortex. The cerebellum was severely atrophied, with granule-cell degeneration, proliferation of astrocytes, and thinning of the myelin. Atrophy in the cerebral cortex was most pronounced in the calcarine cortex (primary visual cortex) and in the precentral gyrus (primary somatosensory cortex). Neurological impairments in infants and children include severe psychomotor retardation, progressive microencephaly, and persistence of primitive reflexes, hyper-reflexia, hypersalivation, and incontinence. Additional motor signs include spasticity, hyperkinesia, ataxia, generalized tonic convolutions, and myoclonic jerking. Visual disturbances range from constriction of the visual field to blindness (O'Kusky, 1992).

The mechanisms of MeHg toxicity have been extensively investigated in experimental models, both in vivo and in vitro. These studies indicate that MeHg act on diverse targets and that neuronal death is caused by more than one mechanism. There is evidence that several cellular functions are impaired by MeHg, including intracellular Ca^{+2} (Minnema et al., 1989) and glutathione (GSH) homeostasis, as well as maintenance of the mitochondrial membrane potential (Sarafian, 1996; Shenker et al., 1999).

Several studies have suggested oxidative stress as one of the major mechanisms involved in MeHg-induced neurotoxicity (Sarafian, 1996). MeHg depletes intracellular GHS, through inhibition of cysteine uptake in astrocytes (Ou et al, 1999; Shanker et al., 2001). Several antioxidants such as selenite, vitamin A and C, catalase (Sanfeliu et al., 2001), estra-1, 3, 5 (10),8-tetraene-3,17α-diol (J-811),

17β-estradiol (Daré et al., 2000), troxol (6-hidroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), and n-propyl galate (PG), a free radical scavenger and superoxide dismutase (SOD) (Shanker and Aschner, 2003), display a neuroprotective effect against MeHg neurotoxicity.

On the other hand, MeHg perturbs a number of cellular processes that most certainly include astrocytic failure to maintain the composition of extracellular fluid. The adequate function of astrocytes is very important, since their functions include neurotrophic factor secretion, control of extracellular pH and ionic balance as well as uptake and metabolism of neurotransmitters, including the excitatory amino acid glutamate. The preferential damage induced by MeHg to astrocytes offers a potential explanation for its neurotoxicity. MeHg is concentrated in these cells and rapidly induces astrocytic swelling. A number of resents studies involvement astrocytic swelling (Aschner et al., 1998), phospholipase A2, glutathione and glutamate in MeHg-induced neurotoxicity (Aschner, 2000; Shanker et al., 2002). These findings contribute for the understanding of the neurotoxicity by MeHg.

2. Glutamate and excitotoxicity.

The amino acid L-glutamate is considered the major mediator of excitatory signals in the mammalian CNS and is probably involved in most aspects of normal brain function, including cognition, memory and learning. Also, it plays major roles in the developing CNS, including synapse induction and elimination, cell migration, differentiation and death. Most neurons and even glial cells have glutamate

receptors in their plasma membranes (Kho, 1991). Further, glutamate is a transmitter substance also in peripheral organs and tissues as well as in endocrine cells. The brain contains huge amounts of glutamate (about 5-15 mmol per kg wet weight, depending on the region), but only a tiny fraction of this glutamate is normally present extracellularly (outside or between the cells). The concentrations in the extracelluar fluid and in the cerebrospinal fluid (CSF) are normally around 3-4 μM and 10 μM, respectively (Hamberger et al., 1983; Lehmann et al., 1983).

Glutamate is continuously released from cells and immediately removed from the extracellular fluid. It exerts its signaling role by action on glutamate receptors, which are located on the surface of the cells expressing them. Therefore, the glutamate concentration in the surrounding extracellular fluid determines the extent of receptor stimulation. It is of critical importance that the extracellular glutamate concentration is kept low, since excessive activation of glutamate receptors is harmful, and glutamate is thereby toxic in high concentrations. In addition, for economy reasons it is necessary to conserve the glutamate released. Intracellular glutamate is considered non-toxic, but it should be keep in mind that intracellular glutamate may not be completely inert (Danbolt, 2001).

Three different families of glutamate receptor protein have been identified. One family of glutamate receptors is activated by the glutamate analogue N-methyl-D-aspartate (NMDA) and these receptors (NR1, NR2A, NR2B, NR2C and NR2D) are collectively referred to as NMDA-receptors. Another family of receptors is activated

by α-amino-3hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and by kainate (Hollmann and Heinemann, 1994). The NMDA and AMPA/kainate are ion channels (conducting only Na^+ or both Na^+ and Ca^+) and are collectively referred to as ionotropic glutamate receptors. The third family of glutamate receptor consist of G-protein coupled receptors, the so-called metabotropic receptors (mGluR1-8) which are subdivided into groups I, II and III. Group I receptors are coupled to phospholipase C and thereby to inositol triphosphate and diacylglycerol production, whereas group II and III are negatively coupled to adenylate cyclase.

Activation of ionotropic glutamate receptors has been shown to increase energy consumption and lead to influx of Na^+ and Ca^{+2}, which have to be pumped out again in a process requiring energy. It therefore also makes sense that neurons became more vulnerable to glutamate after energy deprivation (Sánchez-Carbente and Massieu, 1999)

Excitatory amino acid receptor-mediated neurotoxicity (excitotoxicity) has been proposed to contribute to neuronal loss in a wide variety of neurodegenerative conditions. Considerable evidence implicates NMDA receptor in the processes of excitotoxicity (Danbolt, 2001). This process can cause secondary damage by overexciting nerve cells. Excessive glutamate can damage nerve cells in several ways. One damage pathway starts when an over activation of the glutamate receptor NMDA, opens calcium-permeable channels capable of allowing massive Ca^{+2} influx and also can trigger the release of Ca^{+2} from intracellular stores (Chao, 1995). A number of potential factors leading to cell death might be activated by

severe Ca^{+2} elevations. For example, Ca^{+2}-activated proteolytic enzymes, like calpains, can degrade essential proteins. Moreover, Ca^{+2}-calmoduline kinase II (CaM-KII) is activated, and a number of enzymes are phosphorylated, which increases their activity. Furthermore, Ca^{+2}-dependent endonucleases can degrade DNA. In addition, mitochondria have an important role in the regulation of intracellular calcium concentration. An increased entry of Ca^{+2} into the mitochondria is believed to enhance the mitochondrial electron transport, by increasing the production of reactive oxygen species (ROS) (Dunchen, 2000; Ward et al., 2000).

All these mechanisms, together with enhanced oxidative stress, can induce cell death through necrosis as well as apoptosis, a type of programmed death. Apoptosis is a physiological process during development (Clark, 1990; Ferrer et al., 1992); it can also be induced in adult brain through traumatic injury (Rink et al., 1995), other brain insults (Nicotera et al., 1996; Ferrer et al., 1995), or as a possible mechanism to eliminate cells from inflammatory brain lesions.

Astrocytic glutamate uptake is a potential mediator of mercury neurotoxicity (Aschner et al., 1995, 2000). MeHg inhibits glutamate uptake in cultured astrocytes at low micromolar concentrations (Brookes and Kristt, 1989; Kim and Choi, 1995), and increases D-aspartate release (Aschner and Lo Pachin, 1993). In parallel, it is well established in vitro, that the chronic low-level inhibition of glutamate uptake results in neuronal death (Rothstein et al., 1993). More recently, it was found that MeHg in synaptic vesicles, decreased [3H] glutamate uptake involving the

H+ATPase activity. Until now, the toxic effects of MeHg on CNS were attributed mainly to an impairment of glia glutamate transporters (Porciuncula et al., 2003)

3. MeHg and apoptosis

The cell degeneration in the CNS in MeHg intoxication could be induced by various pathways leading to apoptosis. Cerebellar granule cells are a sensitive target for MeHg neurotoxicity, when are treated with lower doses that MeHg showed morphological changes characteristic of apoptosis (Kunimoto, 1994; Nagashima et al., 1996). Also, MeHg inhibits the migration of cerebellar granule cells in model system for neural development. The impaired migration was a possible cause of the apoptotic death of external granule cells (Kunimoto and Suzuki, 1997)

More recently, it was found that MeHg caused a significant increases in the number of apoptotic cells, but exclusively in immature cultures of fetal rat telencephalon (Monnet-Tschudi, 1998). It has been demonstrated in vitro, that exposure to mercury in the micromolar range leads to apoptotic neuronal death (Castoldi, 2000).

The mechanisms through which MeHg produce apoptosis have been explored in lymphoid cells. Shenker and collaborators (2000) found a significant increase in the presence of cytochrome-*c* in the cytosol of these cells after exposure to MeHg, and the translocation of cytocrome *c* to the cytosol has been show to trigger the downstream apoptotic cascade (Coyle and Puttfarcken, 1993). Specifically, cytosolic cytochrome *c* is known to activate cysteine-aspartate-specific proteases

(caspases) that are constitutively present in most cells, residing in the cytosol as inactive proenzimes. Activation of this family of proteases is responsible for much of the cellular destruction and morphogenic alterations associated with apoptosis. Nishioku et al. (2000) provided the first evidence that MeHg, at relatively low concentrations, induces apoptosis in primary cultured rat microglia, predominately by the caspase-mediated and partially by the endosomal/lysosomal system-mediated mechanisms (Daré et al., 2001).

4. Hypothesis and objectives

The above mentioned antecedents point out to an unbalance of glutamatergic neurotransmission as a possible cause of MeHg toxicity. Our hypothesis is that very low doses of MeHg produce increases in the glutamate extracellular concentration in vivo, which in turn induce neuronal and astrocytic damage. This study will focus on to the evaluation of functional parameters of the CNS in the range of concentrations that do not provoke over toxicity, but that represent the main concern about human health effects of low-level MeHg exposure. We want to analyze in vivo glutamate extracellular levels and its possible participation in brain damage assessed though morphological, behavioral and apoptotic markers.

We can undertake this study using the rat as animal model because numerous studies have allowed to establish comparisons among human and animals, concerning qualitative and quantitative features of neuropathological and neurobehavioral effects of MeHg exposure (Burbacher et al., 1998). These comparisons reveal similarities in neuropathological effects of MeHg on humans and animals at different exposure levels.

The objectives of this work are:

1. To analyze the effects of the MeHg in micromolar range on glutamate extracellular levels in the rat cortex in vivo.
2. To determine whether the MeHg concentrations that induce changes at glutamate levels lead to alterations in the astrocyte, using the inmunohystochemical staining of the glial fibrilar acidic protein (GFAP).
3. To evaluate the behavioral performance of rats in the water maze, after stereotaxic application of MeHg in the hippocampus.
4. To determine whether the MeHg concentrations that induce changes of glutamate extracellular levels also induce apoptosis.
5. To determine the involvement of NMDA receptors in the apoptotic process induced by in vivo exposure to MeHg, using known concentrations to produce significant increases of extracellular glutamate.

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II. Artículo No. 1

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Methylmercury increases glutamate extracellular levels in frontal cortex of awake rats

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Abstract

A current hypothesis about methylmercury (MeHg) neurotoxicity proposes that neuronal damage is due to excitotoxicity following glutamate uptake alterations in the astrocyte. By sampling from a microdialysis probe implanted in the frontal cortex of adult Wistar rats, we measured the effects of acute exposure to either 10 or 100 μM MeHg through the microdialysis probe, on glutamate extracellular levels in 15 awake animals. After baseline measurements, the perfusion of MeHg during 90 min induced immediate and significant elevations in extracellular glutamate at 10 μM (9.8-fold, $P < .001$) and at 100 μM (2.4-fold, $P = .001$). This in vivo demonstration of increments of extracellular glutamate supports the hypothesis that dysfunction of glutamate neurotransmission plays a key role in MeHg-induced neural damage.

Keywords: Methylmercury; Glutamate; Mercury; Excitotoxicity; Microdialysis

1. Introduction

The target site of mercury is the central nervous system (CNS), where different mercury compounds (organic and inorganic) alter parameters related to synaptic function producing diverse behavioral and neuropathological effects [6,9,22]. The molecular mechanisms whereby mercury causes CNS damage have remained elusive, however, a number of recent reports convey important information towards the understanding of methylmercury (MeHg) neurotoxicity.

In vitro, it has been demonstrated that MeHg can affect neuronal and astrocytic function in many different ways. The major mechanisms involved in MeHg neurotoxicity currently explored are the generation of oxidative stress [26,32], disruption of intracellular calcium homeostasis [11,12,16,34] and interference with membrane transport, especially inhibition of glutamate uptake by astrocytes [3,5,17].

Neuronal excitotoxicity following glutamate uptake alterations in the astrocyte is a hypothesis of mercury-induced neuronal damage that has been gaining support through different experimental approaches [2]. It is well established in vitro, that the chronic low-level inhibition of glutamate uptake results in neuronal death [24,31], possibly through cellular processes subsequent to increases of intracellular calcium and reactive oxygen species (ROS) production elicited by the prolonged action of glutamate at postsynaptic receptors [25,30]. In this respect, the participation of MeHg on each of the steps leading to excitotoxic neural damage has been demonstrated. MeHg increases the release of calcium from intracellular organelles [11,12,16] and blocks calcium influx through multiple calcium channel subtypes [34], which modifies intracellular calcium concentrations and neurotransmitter release [20]. In addition, MeHg has high affinity for thiols, which results in the depletion of intracellular glutathione leading to accumulation of ROS [26,27]. Oxidative stress by itself inhibits the astrocytic glutamate uptake mechanisms through a direct action on the transporter proteins [37,38], and it has been recently shown that the inhibition of excitatory amino acid transport induced by MeHg in cultured astrocytes can be reversed by lowering the levels of

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ROS by means of catalase [1]. Neuronal death, necrotic or apoptotic, observed with micromolar concentrations applied to granular cerebellar cells [7] or after subchronic oral administration of MeHg [23], could result from a combination of these alterations.

It is an important piece of information to know whether MeHg is able to increase glutamate concentrations in the brain in vivo. An important difference between in vivo and in vitro conditions in this case is the simultaneous presence and relative integrity of both cellular elements capable of regulating extracellular glutamate levels, namely neurons and astrocytes [10,15]. Although the inevitable development of gliosis at the microdialysis site represents a disturbance of the system, the validity of the microdialysis technique to observe in vivo glutamate fluctuations in response to conditions such as hypoxia and ischemia is supported by numerous studies [4,8,13]. Therefore, we used this technique in order to measure the acute effects of MeHg on glutamate levels in the cortex of awake rats.

2. Materials and methods

Female Wistar rats, bred in house and weighing 230–260 g, were used in these experiments according to the "Guidelines for the Use of Animals in Neuroscience Research" by the Society of Neuroscience. The animals were anesthetized with xylazine–ketamine and placed in a stereotaxic apparatus, the skull was exposed and a hole was drilled for placement of a guide cannula on the frontal cortex (stereotaxic coordinates: AP: −1.0 mm, L: 2.5 mm, at 45° from the vertical). The cannula was fixed to the skull with anchor screws and acrylic cement. After the surgery, rats were individually housed during a 48-h recovery period.

For the microdialysis experiments, a concentric probe (2 mm membrane length, Bioanalytical System, West Lafayette, USA, recovery 25 ± 0.2%) was inserted into the guide cannula. The dialysis probe was perfused at a flow rate of 2 μl/min with a solution containing 147 mM NaCl, 4.0 mM KCl and 1.2 mM $CaCl_2$, pH 7.4. Sample collection was performed every 30 min and started 1 h after the beginning of the perfusion. After three baseline samples, 10 or 100 μM MeHg prepared in perfusion solution that contained exactly the same concentration of NaCl, KCl and $CaCl_2$ than the previous one were infused during the next three samples (90 min). The initial solution was restored for the last three samples. The change of solutions is performed by means of a switch that allows the continuous flow of the perfusion solution through of the probe.

For amino acid quantification, the samples were prederivatized with *O*-phtaladehyde (5 mg OPA in 625 μl methanol, 5.6 ml 0.4 M borate buffer, 25 μl 2-mercaptoethanol, pH 9.5) and separated by means of HPLC using a C18 reverse-phase column (Alltech Associates, Deerfield, IL) with fluorometric detection (Perkin-Elmer, San Jose, CA, USA). The mobile phase was a 50 mM sodium acetate buffer containing 1.5% tetrahydrofuran pH 5.9 and the elution was performed by means of a linear methanol gradient. An external standard was used to construct a calibration curve for glutamate and the samples were diluted so that readings fell within the lineal range. The concentration of glutamate obtained from each sample was expressed in micromolar units.

For the statistical analysis, we performed a mixed multivariate analysis using the following fixed factors: time (1–9), period (basal, exposure and postexposure for each dose) and a random factor of animal (1–15). Using R software (from The R Development Core Team, version 1.4.1, 2002), we performed a linear mixed model fitted by REML, according to Pinheiro and Bates [28]. The contrast function contr.sdif [36] was applied to distinguish between the different factors averages.

At the end of the microdialysis sampling, the rats were sacrificed, the brain removed and fixed in phosphate-buffered formalin. For the histological verification of the placement of the probes, 40 μM thick frozen slices were stained with cresyl violet and examined microscopically. The criteria to include the data collected from a given animal in the subsequent analysis were the absence of hemorrhage and the correct placement of the probe within the frontal cortex of the rat.

The reagents were purchased from Sigma (St. Louis, MO), Caledon Lab. (Georgetown, Ontario) and MeHg from Alfa Aesar (Ward Hill, MA).

3. Results

Fig. 1 shows cresyl violet stained coronal sections through the cortex of a rat implanted with a microdialysis probe. Because of the angled trajectory, the probe crossed through the frontal cortex. The extent of the gliosis along the path of the probe as indicated by the darkly stained cellular elements around the orifice varied among animals. However, in most cases, the degree of local trauma was as shown in Fig. 1A, while the site of insertion of the probe could present a damage as severe as presented in Fig. 1B. In the experiments included in this study, the probe was confirmed to have been placed within the frontal cortex.

The exposure to MeHg provoked significant increases of extracellular glutamate at both concentrations tested. The pattern was different for each animal, but in all cases glutamate levels were higher during MeHg exposure. The mixed effects statistical analysis showed a significant effect of the MeHg exposure [$F(5,108) = 16.2$, $P < .001$].

Fig. 2 shows the temporal course of glutamate release in eight rats exposed to 10 μM MeHg during Samples 4–6. Post-hoc contrasts show a significant difference between the basal and the MeHg exposure conditions ($P < .001$). The postexposure (Samples 7–9) were not significantly different from the exposure levels ($P = .13$). The mean basal glutamate concentration for this experiment was 2.48 ± 0.57,

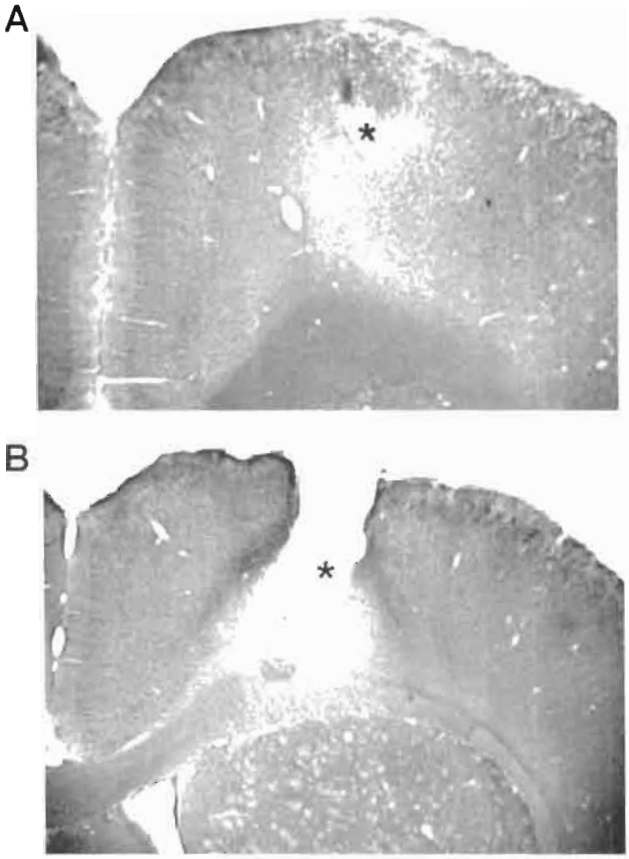

Fig. 1. Photomicrographs of 40 μm coronal sections from the brain of a rat implanted with a microdialysis probe, stained with cresyl violet. The sites of the lesion are marked with asterisks. (A) Trajectory of the probe at AP −0.8 mm relative to bregma. (B) Site of insertion of the probe (AP 1.2 mm).

24.3 ± 7.06 during exposure and 16.11 ± 5.21 μM after the exposure.

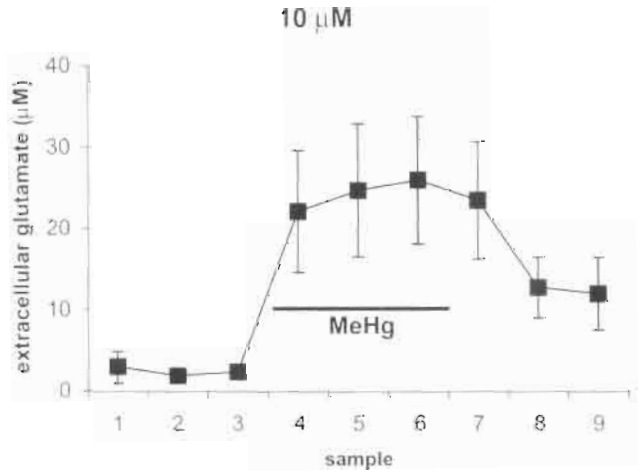

Fig. 2. The mean (± S.E.M.) glutamate concentration obtained from 30 min samples of dialysate in eight animals. Each point represents the mean ± S.E.M. from six to eight samples. Samples 1–3 were basal values. Samples 4–6 represent the exposure period to 10 μM MeHg in the perfusion fluid and Samples 7–9 the postexposure period. The values plotted were not corrected for recovery.

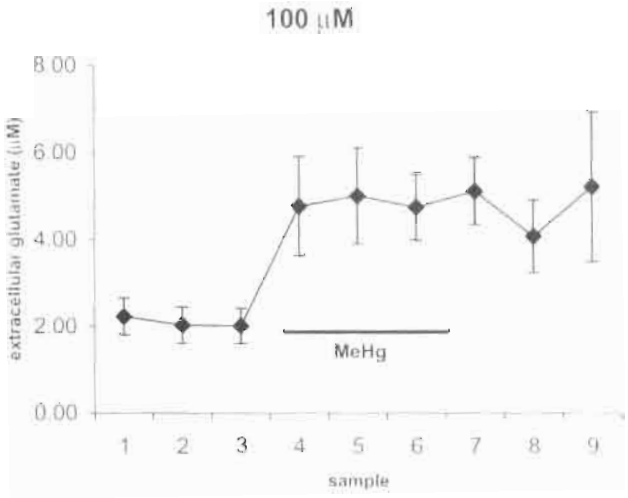

Fig. 3. Time course of extracellular glutamate before (Samples 1–3), during (Samples 4–6) and after (Samples 7–9) 100 μM MeHg exposure through the microdialysis probe in seven animals. Each point represents the mean ± S.E.M. from six to eight samples. The collected fractions (30 min each) were analyzed by HPLC with fluorometric detection for glutamate content. The values plotted were not corrected for recovery.

The pattern of response of seven animals to 100 μM MeHg is presented in Fig. 3. The increment of extracellular glutamate under MeHg exposure was significantly higher than in the basal condition, but not as high as that observed under 10 μM MeHg. Post-hoc contrasts showed a significant difference between the basal and the MeHg exposure conditions ($P = .001$), but not between the exposure and the postexposure condition ($P = .71$), which means that, after the exposure, glutamate levels remain elevated at least during the 90 min following exposure either at 10 or 100 μM MeHg. Basal glutamate levels in this experiment were not different from those of the rats exposed to 10 μM MeHg, namely 2.09 ± 0.57 μM. During MeHg exposure, it reached 5.08 ± 0.97 μM and continue at 4.81 ± 1.11 μM after exposure.

4. Discussion

The present study demonstrates that the acute exposure of the cerebral cortex of freely moving animals to MeHg results in significant increases of the extracellular concentration of glutamate. The relevance of this in vivo assessment of glutamate levels is that it shows the resultant of several affected functions, previously explored in vitro, on one tightly regulated variable, namely glutamate extracellular levels.

In vitro, exposure to 10 μM MeHg during 10 min has been shown to produce 50% inhibition of glutamate uptake in rat astrocytes [17] that was not accompanied by cytotoxicity. At longer exposure periods (1–3 days), however, human astrocytes in culture begin to show signs of cytotoxicity and mortality [32]. By contrast, neurons are much more vulnerable to the toxic effects of MeHg. In vitro, continuous

exposure to 10 μM MeHg during several hours results in 100% neuronal death in cell cultures from human, rat and mouse [7,26,32]. We wanted, therefore, to know whether the levels of extracellular glutamate at this MeHg concentration were increased in vivo, when astrocytes are expected to be relatively intact but with a decreased rate of glutamate uptake. On the other hand, the higher concentration tested (100 μM) has been reported to completely suppress action potentials in rat hippocampal slices [39], it is also in the range of concentrations that suppress sodium and potassium currents in neuroblastoma cells [29] and there are no reports of in vitro glutamate uptake inhibition at this MeHg concentration. Adult rats orally exposed during 20 days to MeHg exhibit hind leg paralysis and have an approximate brain Hg concentration of 100 μM [23]. From these antecedents, we expected a decreased neural activity and greater cytotoxicity when the rat cortex is exposed to 100 μM MeHg, therefore, we wanted to compare the in vivo levels of extracellular glutamate under a low- and a high-exposure to MeHg. It should be noticed that MeHg is not delivered totally to the cortical tissue, but circulated through the microdialysis probe, which would result in a local infusion of about 2.5 and 25 μM, respectively. Given that MeHg is assumed to remain and diffuse in the tissue, the highest concentrations that could be built up in this experiment were 10 and 100 μM.

We have found that acute exposure to MeHg increases glutamate extracellular concentrations 2.4-fold at 100 μM and 9.8-fold at 10 μM. Brain injury and generation of oxidative stress is associated with 2.8-fold increases of extracellular glutamate [13], and the local glutamate concentrations that we observed can be associated with production of hydroxyl radical [8]. We think that these increased concentrations in the synaptic space may contribute to progressive dysfunctions of the neuron and astrocyte. We interpret the increase in glutamate levels at 10 μM MeHg as a result of several events: energy deficits in astrocytes and neurons leading to reduced uptake mediated by ROS, an intracellular calcium concentration elevation due to overstimulation of glutamate receptors that also could produce ROS, as well as an increased glutamate release due to membrane depolarization and deficient calcium buffering. By contrast, at 100 μM, there is a substantial inhibition of sodium, potassium and calcium channels by MeHg, which could result in lower release rates than in the previous situation, although ROS and the subsequent uptake inhibition are still present due to effects on the mitochondria and disturbances of calcium homeostasis [35]. Extracellular glutamate under these experimental conditions remains significantly elevated during 90 min after the exposure to MeHg due to prolonged or perhaps irreversible changes in extracellular glutamate regulation.

The relevance of this study to human exposure could be marginal, given that neurotoxicity of MeHg is consequent to chronic ingestion of contaminated food, not to an acute, direct brain exposure to this compound. However, the range of concentrations tested in this study are not far away from those that can be found in the brain of exposed humans. It has been proposed [6,18] that the brain Hg concentration can be estimated from blood or hair Hg levels and, according to these estimates, concentrations ranging from 2.5 to 10 μM are associated with delayed psychomotor development in children and in exposed adults with minimal signs of MeHg poisoning [14], while children with severe disabilities (Minamata disease) had estimated brain concentrations during development ranging from 35 to 70 μM and can be estimated as high as 12 μM several years after birth [21].

Since excitotoxicity is a multifactorial process, the relationship between accumulation of extracellular glutamate and neuronal cell death is not necessarily direct [19]. In vivo, it has been recently demonstrated that substantial elevations of extracellular glutamate result in neuronal damage only when accompanied by an energy-deficit, confirming the link between mitochondrial dysfunction and glutamate excitotoxicity [33]. We believe that the capacity of MeHg to impair mitochondrial metabolism and this in vivo inhibition of glutamate uptake plays a key role on its neurotoxic properties.

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III. Artículo No. 2

EFFECTS OF LOCAL INFUSION OF METHYLMERCURY ON THE RAT BRAIN: GFAP IMMUNOHISTOCHEMISTRY AND WATER MAZE LEARNING.

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INTRODUCTION

Several recent reports in the literature support a role of the neurotransmitter glutamate in methylmercury (MeHg)-induced neurotoxicity. When MeHg concentrations in the micromolar range are directly applied to the cortex of freely moving rats, several-fold increases in extracellar glutamate are observed in microdialysates [1]. According to *in vitro* studies, the increase of extracellular glutamate reflects a blockage of its reuptake by the astrocyte [2]. As a result, the prolonged action of glutamate at postsynaptic receptors can lead to apoptotic or necrotic cell death [3,4], specially when exposure takes place during development. Miyamoto and collaborators [5] demonstrated recently that during the early postnatal development the sensitivity of the NMDA receptor is enhanced leading to an increased production of cytotoxic free radicals. This enhanced sensitivity is attributed to the augmented expression of the NR2C subunit of the NMDA receptor at this stage, which is insensitive to Mg^{2+} blockade [6].

Although excitotoxicity is expected after MeHg exposure, it is not know whether this event provoke a permanent damage to the central nervous system (CNS) or it is repaired through the plastic mechanisms available even in the adult brain. It is well documented that in cases of human exposure to MeHg, although pregnant mothers did not show CNS impairment, their children were born with severe brain damage [7,8]. It is therefore necessary to link the observed neurochemical changes in rats to cellular of subcellular elements in the CNS in order to

characterize MeHg-induced damage. Also, given the reserve capacity of the brain to counteract from subtle to extensive damage, the functional assessment of the whole animal contributes to determine the impact of a restrained chemical lesion to the complex output of the CNS.

For this purpose, we undertook a series of immunohistochemical and behavioral experiments in animals exposed in vivo to a single dose of MeHg through stereotaxic injection in the cortex and hippocampus, using concentrations known to produce significant increases of extracellular glutamate [1]. Since MeHg is a prototype neurotoxicant [9] that increases the immunohistochemical staining of the glial fibrillar acidic protein (GFAP), we used this technique and the water maze test, which detects functional alterations of the hippocampal formation after exposure to a wide range of toxicants [10-11].

METHODS

Female Wistar rats (200-250g) were anesthetized with xylazine-ketamine and placed in a stereotaxic apparatus. Two MeHg doses and 2 periods of time after infusion were tested, therefore, 6 groups of 4 rats each were prepared for immunohistochemistry, and 6 groups of 5 animals each for the behavioral tests. Through a hole drilled in the skull 3.2 µl of a solution containing either saline, 1.2 or 12 nmol MeHg were slowly injected in the frontal cortex (stereotaxic coordinates AP: 0.1, ML 0.25, DV -0.2) or the hippocampus (AP –0.38, ML 0.23, DV 0.3), according to Paxinos and Watson [12].

For GFAP determinations the animals were deeply anesthetized at 7 or 14 days after MeHg infusion and perfused through the hearth with saline and paraformaldehyde and the brain extracted. Frozen coronal sections (40 μM) were obtained and immunostained for GFAP . The images were digitalized and the optical density compared between MeHg and saline infused animals. The non-lesioned hemisphere was used as internal control for the quantification of immunostaining in each slice.

The behavioral testing in the watermaze of the animals that were injected in the hippocampus took place during 7 consecutive days, starting 3 days and 9 days after MeHg infusion, in order to evaluate the animals during the first and the second week post-lesion, respectively. The watermaze was a 2 m diameter pool constructed according Morris and collaborators [10]. The pool was filled with water made opaque by adding a small amount of milk. The escape platform was a 10 cm diameter plexiglass surface hidden 2 cm beneath the surface of the water and fixed in one of the quadrants of the pool. Four trials were given to each rat per day and 3 parameters were considered in order to evaluate performance in this task: escape latency, number of failures to reach the platform/number of trials and number of times crossing the tank/minute, as previously reported [13].

The statistical significance of the results was determined through ANOVA for the parametrical data, and through Kruskal-Wallis followed by Mann-Whitney for the non-parametrical data (failure rate and number of times crossing the tank/minute).

RESULTS

Examples of GFAP-immunostained slices for each treatment are shown in figure 1. One week after MeHg or saline infusion a more intense glial reaction was observed in the MeHg-treated animals (Fig 1B and 1C) than in controls (Fig 1A). The quantitative analysis of optical density revealed 6 % and 16% increase of GFAP staining in the 1.2 nmol and in the 12 nmol-group, respectively ($p = 0.06$), while the same analysis performed in the animals studied 2 weeks after MeHg administration did not show any significant effect (Fig 1 D,E and F).

The mean latencies to find the hidden platform in the watermaze test are shown in the figure 2 . Both groups exposed to MeHg presented similar escape latencies to those of the control group. When tested during the first week after the lesion, controls decreased the escape latency from 237± 64 s on the first day to 59 ± 81 s on the seventh day, the 1.2 nmol-group from 135 ± 112 to 45 ± 43 and the 12 nmol-group from 209 ± 69 to 68 ± 68. For all 3 groups, latencies were shorter in the animals tested the second week after the surgery as shown in fig 2 B, but no significant differences among them were found. The number of times crossing the tank/ min as well as the number of failures to reach the platform were not significantly different among treated and control groups (data not shown).

DISCUSSION

Methylmercury, as a potent neurotoxicant, has been shown to affect several of the parameters of neurotransmission on which it has been tested. MeHg interferes with such diverse processes as neurotransmitter release and uptake,

mitochondrial function, membrane transport, enzymatic activity and protein synthesis, to mention only a few. Therefore, in order to asses the relevance that excitotoxicity may have as a mechanism of MeHg-induced neurotoxicity, it is necessary to support the neurochemical findings with behavioral and morphological studies. In this context, knowing that the increase of GFAP in response to neurotoxicants is transient, with the time course of the decline varying from toxicant to toxicant [14], we wanted to know whether the doses of MeHg that induce important increases of extracellular glutamate elicit also an important astrocytic response. As expected, 2 weeks after the lesion GFAP immunoreactivity is identical between control and exposed animals, but one week after the lesion, only a tendency to a dose-dependent increase on cortical GFAP is observed (fig 1). This indicates that either important increases of extracellular glutamate in the adult rat does not initiate a robust glial reaction, or that this reaction occurs earlier than one week after the lesion, since gliosis is regarded as a relatively late step in the cascade of events that follows neuronal damage.

It is also plausible that local increases of extracellular glutamate do not produce an important neuronal damage that can not be repaired through the reserve mechanisms of the brain. Our results from the watermaze point in this direction, since we did not observe any difference between the performance of control and exposed animals. This does not imply that the approach is inherently insensitive, but may reflect the reserve capacity of this brain region to absorb the toxic insult without an alteration in behavior [15].

In summary, the adult rat does not develop significant astrocytic reactions nor place learning deficits after the single exposure to amounts of MeHg that may result in glutamate-induced excitotoxicity. These results suggest that the role of glutamate in MeHg induced-neurotoxicity is limited to sensitive periods during CNS development.

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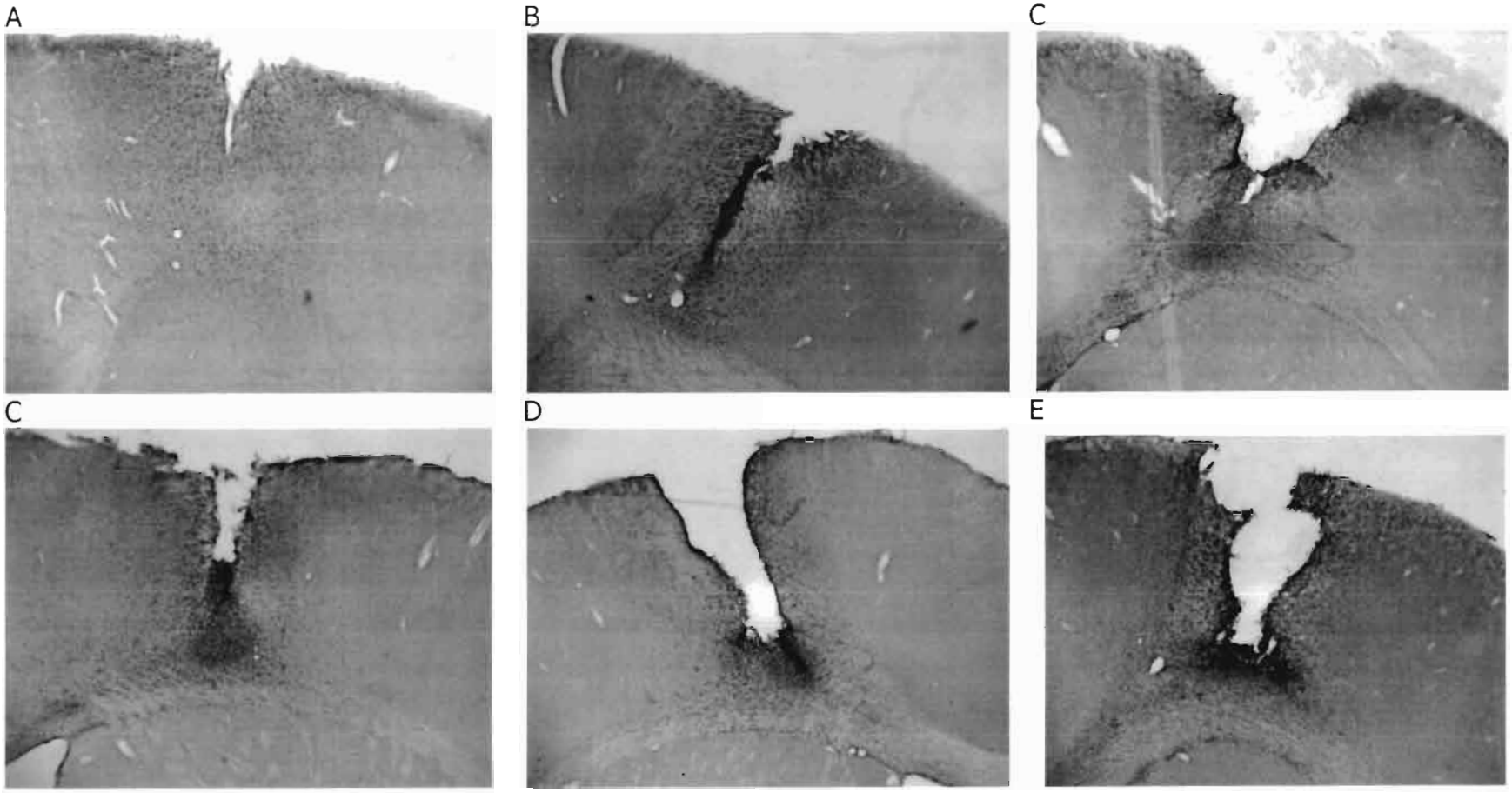
FIGURE LEGENDS

Figure 1. Coronal sections (40 μm) immunostained for GFAP showing the cortex of rats infused with saline (A), 1.2 nmol MeHg (B) and 12 nmol MeHg (C) one week before sacrifice and immunohistochemical staining. The same sequence is followed in the bottom panel for animals sacrificed 2 weeks after the infusion of either saline or MeHg.

Figure 2. . Mean escape latency scored by the experimental groups over the 7 days (28 trials) of water maze testing. Each animal was given 4 trials per day, thus each point represents the mean escape latency that was averaged to yield mean performances per group. Saline -♦-; 1.2 nmol MeHg □; 12 nmol ?.Trials started 3 days (A) and 9 days (B) after MeHg or saline stereotaxic infusion.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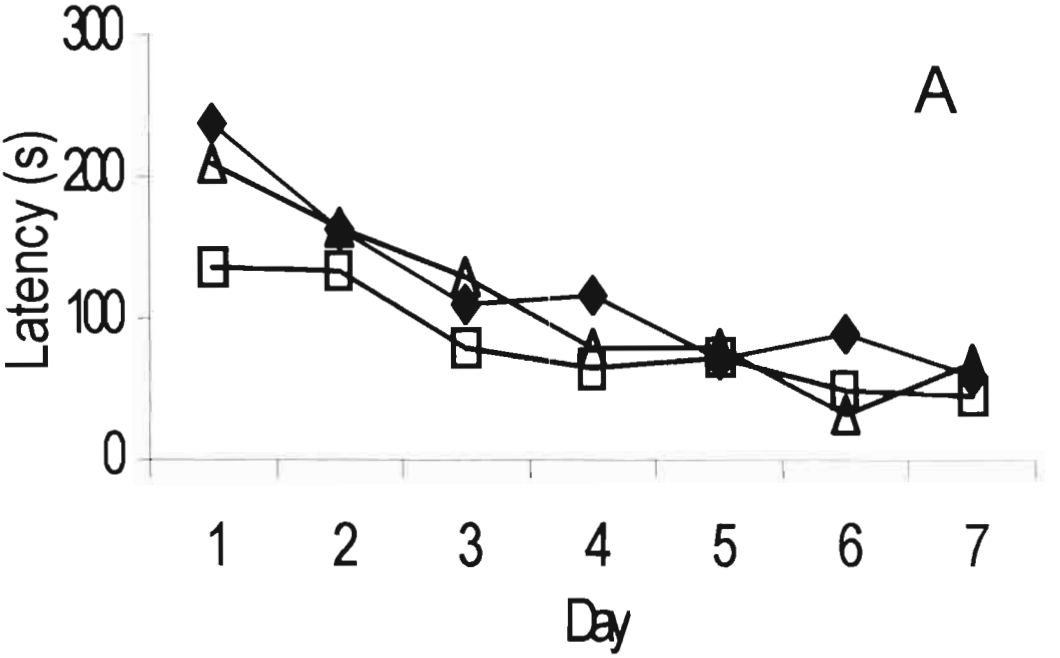
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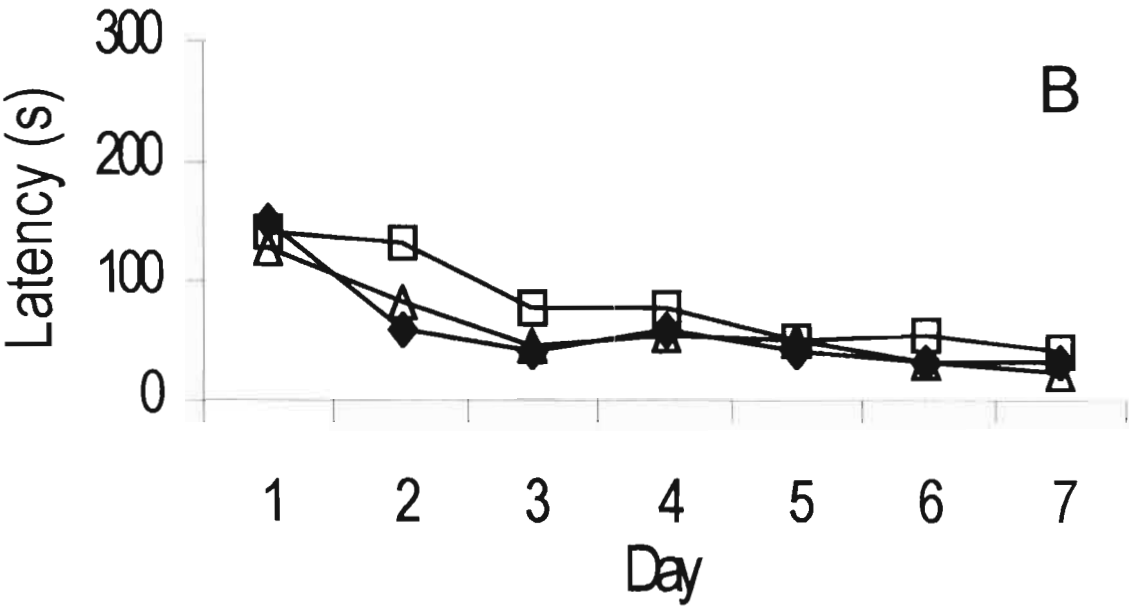
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WESTERN PHARMACOLOGY SOCIETY

46th Annual Meeting, Feb. 2nd – 6th, 2003
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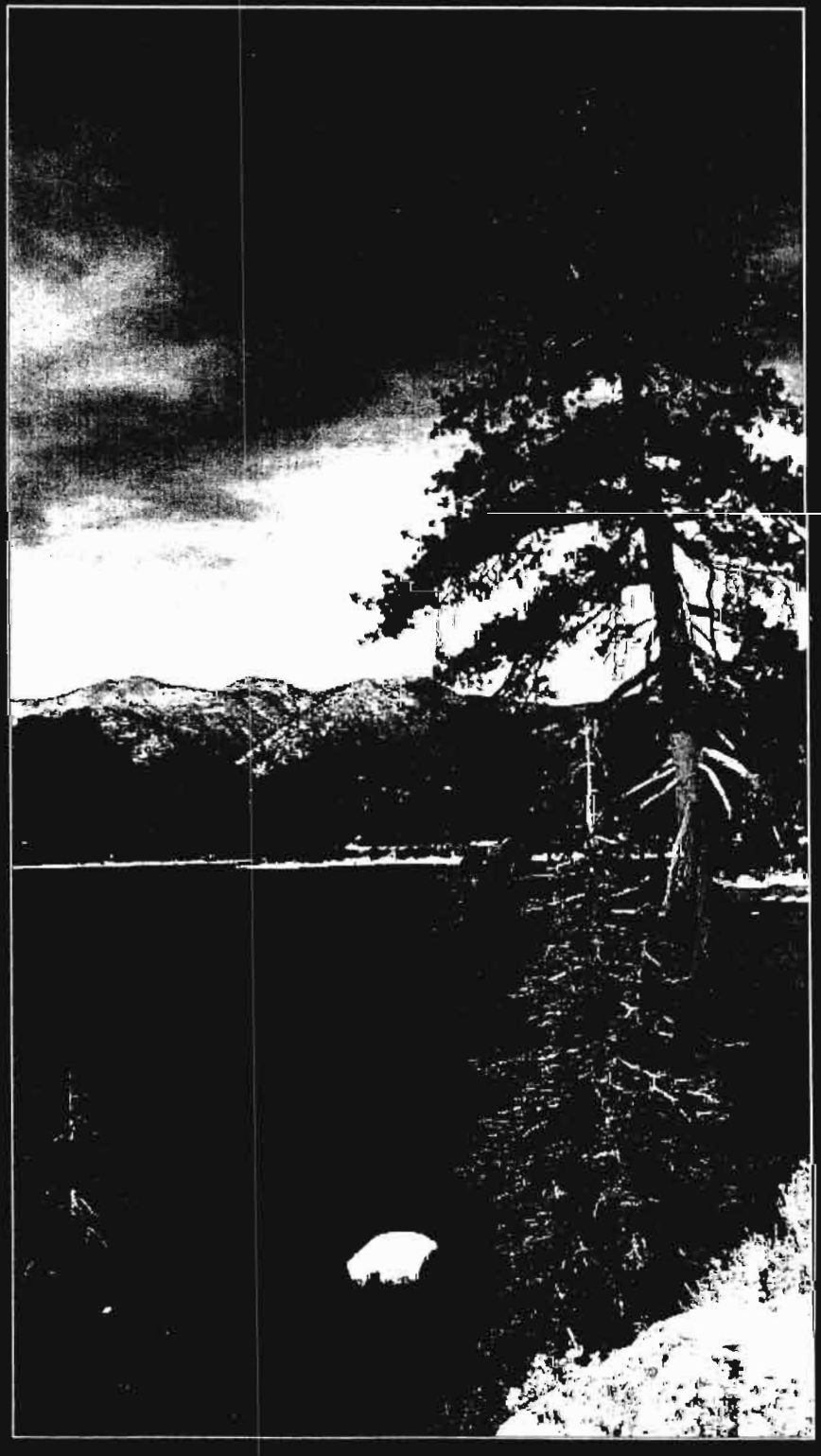

Tahoe 2003

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Meeting Program & Abstracts

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46TH Annual Meeting
February 2nd – February 6th, 2003
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T-37)

PHARMACOKINETICS OF STAVUDINE BY ORAL ADMINISTRATION TO HEALTHY MEXICAN VOLUNTEERS. Y. Escobar, C.R. Venturelli, E. Escobar-Islas and C. Hoyo-Vadillo, Department of Pharmacology, CINVESTAV-IPN, Av. IPN 2508, Mexico city, Mexico. (carlos@mail.cinvestav.mx)

We evaluated the pharmacokinetic of the antiviral drug stavudine. It was given in a single dose of 40mg to healthy volunteers, 9 male and 15 females between 30 and 40 years old. After the oral administration, blood samples were taken at time 0, 15, 30, 45 and 60 min for the following 2, 4 and 6 h after the drug administration. Stavudine was quantified by an specific HPLC method. We found that the Cmax is almost the double of the one reported in the literature (1424.3 ng/ml vs 876.3 ng/ml), the t1/2 is just a bit longer (1.3 h vs. 0.9-1.2 h) and the AUC 0-infinity falls in the reported range (1663.9 h*ng/ml in our study vs the reported range from 1246 to 1945 h*ng/ml).

T-38)

EFFECTS OF LOCAL INFUSION OF METHYLMERCURY ON THE RAT BRAIN: GFAP IMMUNOHISTOCHEMISTRY AND WATER MAZE LEARNING. B. I. Juárez[1], L. M. Martínez[1], C. G. Castillo[2], M. Giordano[2], C. García[1] y M. E. Jiménez-Capdeville[1]. [1]Departamento de Bioquímica, Facultad de Medicina, Universidad de San Luis Potosí, México; [2] Laboratorio de Plasticidad Cerebral, Centro de Neurobiología, Universidad Nacional Autónoma de México, México.

The acute exposure of the rat cortex to methylmercury (MeHg) induces large increases of extracellular glutamate concentrations. According to current hypothesis on MeHg neurotoxicity, brain damage occurs through excitotoxic events, especially during development, when the NMDA receptors are more susceptible to overactivation by glutamate, while in adults those augmentations may be counteracted by regulatory mechanisms. Using immunohistochemistry, we quantified GFAP presence 1 and 2 weeks after the stereotaxic injection in the rat frontal cortex of 1.2 and 12 nmol of MeHg or saline, concentrations which are known to increase extracellular glutamate. Image analysis of the stained slices showed a dose-dependent increase in immunoreactivity 1 week after the lesion, but those increases were not longer present at 2 weeks. In order to explore whether MeHg exposure produces functional alterations, the same amounts of MeHg were injected in the hippocampus, and a specific task for damage in this region, the water maze (6 days), was performed 1 and 2 weeks after the lesion, starting on days 3 and 9 after the surgery, respectively. Latency to reach the platform, number of times crossing the water tank and number of failures were used to evaluate performance. None of the exposed groups showed differences compared to the control group (saline infusion). These results indicate that in adult animals, the increases of extracellular glutamate produce reversible changes of glial reactivity markers in the cortex. If these transitory changes take place also in the hippocampus, they do not result in learning deficits of the water maze task.

IV. Artículo No. 3

PARTICIPATION OF N-METHYL D-ASPARTATE RECEPTORS ON METHYLMERCURY-INDUCED APOPTOSIS IN RAT FRONTAL CORTEX

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Number of text pages: 14

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Abstract

Methylmercury (MeHg) inhibits glutamate uptake by astrocytes, and this could contribute to neuronal loss through excitotoxicity. We explored the extent at which this phenomenon is involved in MeHg –induced apoptosis in the rat cortex. MeHg amounts that increase extracellular glutamate (1.5, 7.5 and 15 nmol) were stereotaxically administered to adult rats before apoptosis determination by means of TUNEL assay. All doses produced significant apoptosis increases. The previous administration of MK-801, a non competitive NMDA receptor antagonist, reduced apoptosis significantly, which demonstrates that excitotoxicity contributes importantly to MeHg neurotoxicity.

Methylmercury (MeHg) is an environmental pollutant that alters the normal structure and function of the central nervous system (CNS), particularly when exposure takes place during development [6]. The underlying mechanisms of MeHg neurotoxicity have been extensively investigated using in vitro and in vivo models, and now we know that it perturbs a variety of cellular functions, such as intracellular Ca^{+2} homeostasis [15, 13, 25], glutathione balance [21], mitochondrial membrane potential [11], control of reactive oxygen species (ROS) generation [7, 29], and glutamate uptake [1, 12] among others. As a consequence of these alterations, in vitro studies have demonstrated, that neurons and glial cells exposed to MeHg undergo cell damage and either apoptotic or necrotic death, depending on the intensity of exposure and the developmental stage of the nervous system [4, 18].

N-methyl-D-aspartate (NMDA) receptors play a crucial role in MeHg neurotoxicity, since their overactivation after MeHg-induced glutamate uptake inhibition can trigger the excitotoxic cascade [5]. The developing CNS is more susceptible to the toxic effects of both N-methyl-D- aspartate and MeHg , due to the reduction of voltage-dependent Mg^{2+} blockage at this stage. This damage can be partially prevented by the administration of MK-801, a non-competitive NMDA-antagonist [16, 17]. Although the NMDA receptor-mediated increase of intracellular Ca^{2+} concentration elicits a series of cellular changes that can lead to apoptosis, MeHg can activate by itself different downstream points of the apoptotic cascade, as mentioned above. Therefore, the extent at which an excitotoxic phenomenon is

involved in MeHg-induced apoptotic cell death has not been fully elucidated. In this respect, in vitro studies employing astrocytes [2, 24, 7, 8], glioma cells [3], microglia [20, 18], and cerebellar granule cells [10, 19, 4] have contributed to characterize the apoptotic pathway induced by MeHg in isolated neural cell types in vitro, however, the in vivo outcome of a sustained glutamate reuptake inhibition by MeHg has not been addressed. Thus, the purpose of this work was to determine the protection provided by the non-competitive NMDA antagonist MK-801 against the induction of apoptosis by in vivo exposure to MeHg through stereotaxic injection in the rat cortex, using concentrations that significantly increase the extracellular glutamate [12].

Experiments were performed in adult female Wistar rats (200-250 g) bred in house. Animals (5-9 per group) were anesthetized (ketamine 100 mg/kg, and xylazyne 8 mg/kg) and received stereotaxic injections of sterile saline or MeHg solutions (1.5, 7.5 and 15.0 nmol in 3.8 µL) into the frontal cortex (AP =+ 0.02 cm; LM =+0.25 cm; DV =-0.4 cm from bregma), according to Paxinos and Watson [22]. Two time-points after injections were selected to asses apoptosis, 24 and 48 h, according to a preliminary series of experiments to trace the time course of the phenomenon. Parallel experiments with animals treated with MK-801 malate (CALBIOCHEM, Darmstadt), (10 mg/kg, i.p.), were performed to analyze the participation of NMDA receptors.

Under deep pentobarbital anesthesia, rats were perfused through the hearth with saline (50 mL) and phosphate buffered saline (PBS, 50 mL). After decapitation, the

brain was extracted and a 3 mm diameter section around the injection site, from the cortex surface to the corpus callosum, was dissected over ice. Immediately, the tissue was mechanically disaggregated in PBS, and 1 x 10^6 cells were isolated for apoptosis assessment (viability was always higher than 99%, evaluated by tripan-blue dye exclusion). Detection of apoptosis was performed by TUNEL using the APO-DIRECT™ staining kit (Phoenix Flow Systems, San Diego, CA), according to the manufacturer's instructions. Brain cells were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA) and the results were expressed as the percent of apoptotic cells. Raw data were log transformed and analyzed by a factorial ANOVA, including MeHg (four levels or doses) and antagonist (two levels, presence or absence) as factors. Pos hoc Fisher test was employed to establish multiple comparisons among groups. A value of $p < 0.05$ was considered statistically significant. All analyses were performed with the software STATISTICA version 6.0.

We first determined the dose dependency of apoptosis rate at both times studied. All MeHg doses tested produced a highly significant increase of apoptosis, at 24 h and 48 h ($p < 0.001$ in both cases). At 24 h a clear dose response effect was observed, increasing from 0.8% of apoptotic cells in the control group to 5.7, 14.9 and 23.7 % at the three doses employed, respectively (Fig. 1). The induction of apoptosis was even higher at 48 h but only for the group exposed to 1.5 nmol MeHg, the groups exposed to the two higher doses of MeHg showed an important decrease of apoptotic cells (Fig 2).

Administration of MK-801 resulted in a significant reduction of apoptosis at 24 h ($p < 0.001$) and 48h ($p < 0.05$), at all doses employed. At 24 hours, the MK- 801

decreased the MeHg-induced apoptosis between 62 and 78% (Fig 1), while at 48 hours the decrement ranged from 30 to 67 %. Despite the considerable protection provided by this antagonist, the levels of apoptosis were still significantly higher than control values when 7.5 and 15 nmol MeHg were employed for 24 h, as well as those of the group exposed 48 hours to 1.5 nmol.

These results demonstrate that direct MeHg application in the rat cortex triggers an apoptotic process, which takes place in a dose-dependent way within 24 hours after exposure. Together with our previous findings [12], the dose dependency of apoptosis upon MeHg exposure supports the hypothesis that cell death is associated with an increase of extracellular glutamate through the inhibition of glutamate reuptake [1]. The finding that 48 hours after exposure the apoptosis percentage falls in the high-dose exposed groups could be related to the multiple sites of action of MeHg downstream the apoptotic cascade [28], which can be simultaneously involved when higher amounts of MeHg are available. The apoptotic process elicited by high MeHg doses, massive within the first 24 hours and decreasing the second day, could be due to the disappearance of Tunel-positive apoptotic cells, a phenomenon that is consequence of their fragmentation, and subsequent forming apoptotic blebs that are phagocytosed by neighboring cells.

The dependency upon NMDA receptor activation for the apoptotic process found in this study indicates that the effect of MeHg is strongly associated with excitotoxicity. However, the inhibitory effect of MK-801 decreased to non-significant levels in rats exposed to 7.5 nmol, and less than 50% of inhibition was observed in

the group exposed to 15 nmol. This suggests a more important participation of other apoptotic mechanisms than NMDA receptor activation under these circumstances. The present experimental method does not allow to identify the cell types that were dying through excitotoxicity, since NMDA receptors are present in both neurons and glia [23]. However, in vitro studies refer that both astrocytes, microglia and neurons die upon MeHg exposure [18], and that a neuron subset, the cerebellar granule cells, is not protected by MK-801 [4]. In addition, these in vivo experiments require the administration of anesthetics, which in the case of ketamine represents also a blockade of NMDA receptors, that could lead to an underestimation of the actual apoptotic rate induced by MeHg.

In conclusion, the present study demonstrates the importance of glutamate toxicity under low-level MeHg exposure. Given its slow elimination rate from brain tissue, environmental exposure to this compound can result in a low but sustained induction of apoptosis, mainly through the cascade that starts with overactivation of NMDA receptors. Other neurotransmitters as dopamine can also participate in this phenomenon, since its extracellular concentration increases under MeHg exposure and this phenomena is prevented by NMDA receptor blockade [9]. The concomitant increase of neuromodulators such as dopamine brings the membrane potential closer to NMDA receptor activation. Although it is described that neurotoxicity elicited by an excess of extracellular glutamate acting at NMDA receptors is simultaneously counteracted by the activation of metabotropic

receptors [14], excitotoxicity still accounts for an important fraction of CNS apoptosis elicited by MeHg exposure.

Acknowledgements

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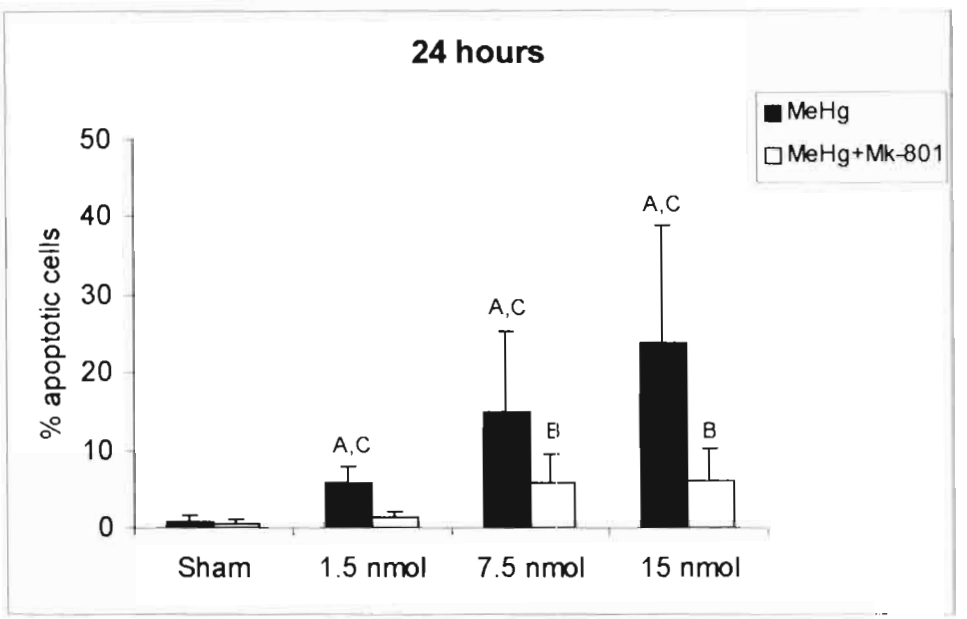
FIGURE LEGENDS

Fig 1. Effect of MeHg exposure on percentage of apoptotic cells in the rat cortex. Twenty-four hours after stereotaxic injection of either MeHg or saline (sham group) apoptosis was determined by means of the TUNEL assay, as described in the text. Black bars represent the mean ± SEM of 5 to 9 animals. The effect of NMDA receptor blockade through previous i.p. administration of MK-801 is presented in the white bars (n = 4-9). ^{A} $p < 0.001$ compared to the sham group, ^{B} $p < 0.001$ compared to the sham + MK-801 group, ^{C} $p < 0.001$ compared with the same dose applied in the presence of MK-801, factorial ANOVA followed by Fisher test.

Fig 2. Effect of 3 doses of MeHg on the apoptosis rate in the cerebral cortex of rats (black bars) and in presence of MK-801 (white bars) 48 hours after exposure. Bars represent the mean ± SEM of 5 to 9 independent experiments. ^{A} $p < 0.001$ compared to the sham group, ^{b} $p < 0.01$ compared to the sham + MK-801 group, ^{c} $p < 0.01$ compared with the same dose applied in the presence of MK-801, factorial ANOVA followed by Fisher test.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7.5 nmol
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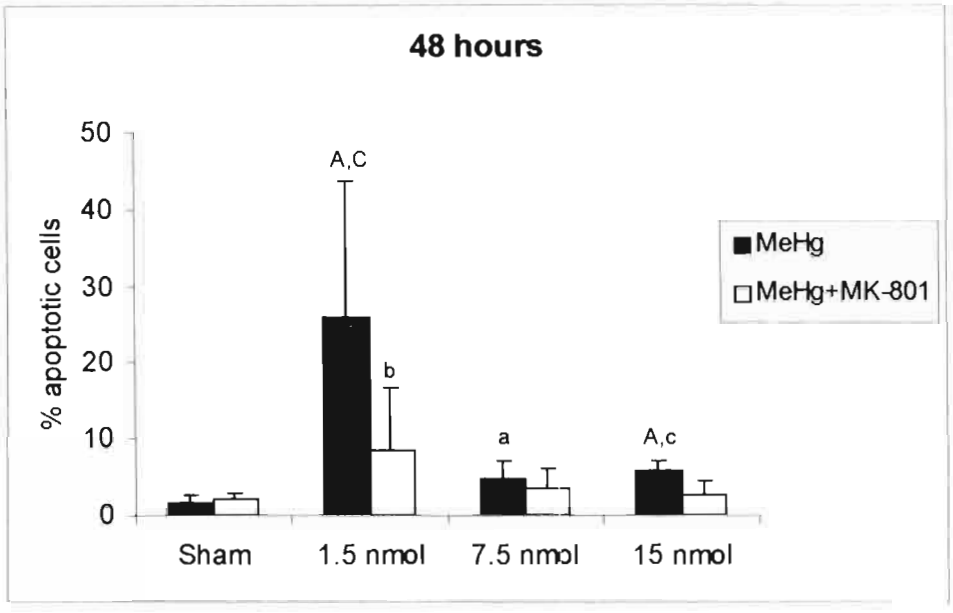
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7.5 nmol
15 nmol

July 29, 2003

Dear Dr. Jiménez-Capdeville:

Your submission, PARTICIPATION OF N-METHYL-D-ASPARTATE RECEPTORS ON METHYLMERCURY-INDUCED APOPTOSIS IN RAT FRONTAL CORTEX, has been received by the Editorial Office and will be processed as soon as possible.

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