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Manganese poisoning reduces strychnine-insensitive glycine binding sites in the globus pallidus of the mouse brain.

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Key words: Manganese toxicity, glycine receptors, autoradiography, [^{3}H]-glycine, globus pallidus.

Abstract. Manganese (Mn) poisoning is characterized by central nervous system manifestations, including psychiatric disturbances and extrapyramidal disorders. This metal is thought to produce neuronal degeneration due to cytotoxic products originated by oxidative stress and through an indirect excitotoxic process. In previous studies, we have found a reduction in the density of N-methyl-D-aspartate (NMDA) recognition sites in some brain areas of Mn-treated mice. Due to the close relationship between NMDA sites and strychnine-insensitive glycine (Gly) modulatory sites in the NMDA receptor complex, the [^{3}H]-glycine ([^{3}H]-Gly) binding was analyzed by autoradiographic methods in the brain of mice treated with manganese chloride for 8 weeks. Among all analyzed areas, only the globus pallidus showed a significant reduction in [^{3}H]-Gly binding (27-28%). The Gly binding decrease, focalized in the globus pallidus, could reflect a degeneration of structures containing strychnine-insensitive Gly receptors, since this area is the most frequently reported damaged brain region in Mn intoxication. However, it might also be due to a Gly receptor down-regulation to control NMDA complex activation during Mn poisoning.

Reducción de los receptores glicinérgicos insensibles a estricnina en el globo pálido del cerebro de ratones intoxicados con manganeso.

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Palabras claves: Intoxicación con manganeso, receptores glicinérgicos, autorradiografía, [^{3}H]-glicina, globo pálido.

Resumen. La intoxicación con manganeso (Mn) está caracterizada por alteraciones en el sistema nervioso central, incluyendo disturbios psiquiátricos y desórdenes extrapiramidales. Este metal produce degeneración neuronal por medio de productos citotóxicos, originados por estrés oxidativo, y a través de un proceso excitotóxico indirecto. En estudios previos se encontró una reducción de los sitios de unión al N-metil-D-aspartato (NMDA) en algunas regiones cerebrales de ratones tratados con Mn. Debido a la estrecha relación estructural y funcional que existe entre los sitios de unión al NMDA y los receptores modulatorios glicinérgicos insensibles a estricnina en el complejo receptor NMDA, se analizó mediante métodos autorradiográficos la unión de la [^{3}H]-glicina en secciones cerebrales de ratones tratados con cloruro de manganeso durante 8 semanas. De las 20 áreas analizadas, sólo el globo pálido mostró una reducción significativa de la unión de la [^{3}H]-glicina (27-28%). La disminución de la unión de la [^{3}H]-glicina, focalizada en el globo pálido, puede reflejar degeneración de estructuras que contienen receptores glicinérgicos insensibles a estricnina, ya que frecuentemente se han reportado daños en esta región cerebral durante la intoxicación con este metal. Sin embargo, no hay que descartar la posibilidad de que dicha reducción sea debida a una regulación hacia abajo del número de receptores glicinérgicos para controlar la activación del complejo receptor NMDA durante la intoxicación con Mn.

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INTRODUCTION

Excessive exposure to manganese (Mn) causes a neurodegenerative disease characterized by behavioral alterations and motor dysfunction, which resembles a dystonic parkinsonian disorder (10, 13, 27). Two mechanisms have been proposed as responsible for the neuronal degeneration produced in this disturbance: 1) an oxidative stress generated by oxygen free radicals, which are produced during the enhanced autoxidation of catecholamines by higher-valence Mn ions (12, 17, 20); 2) an indirect excitotoxic process, secondary to the energy depletion caused by this metal (2, 6). This second mechanism seems to be mediated by N-methyl-D-aspartate (NMDA) receptors, since the neuro-

toxic effects of Mn were blocked by prior removal of glutamatergic input or after treatment with a NMDA antagonist (6).

At the molecular level, the NMDA receptor complex has been demonstrated to consist mainly of a voltage-dependent cation channel with several recognition sites, that function collectively to regulate NMDA receptor-mediated responses. The principal domains are a L-glutamate (Glu)/NMDA recognition site (18), a strychnine-insensitive glycine (Gly) modulatory site (23), a binding site for dissociative anaesthetics, such as phencyclidine (PCP site) (16), and a polyamine recognition site (37).

Complex interactions exist between NMDA binding sites and strychnine-insensitive Gly recognition sites in the NMDA receptor. Gly is known to allosterically potentiate Glu responses by increasing the frequency of channel openings, once Glu or NMDA is bound to the receptor, without changing the unitary conductance or the mean open time (23). Furthermore, NMDA receptor agonists have been reported to stimulate [^{3}H]-glycine ([^{3}H]-Gly) binding whereas antagonists inhibit this binding (25).

Abnormalities of NMDA receptor regulation by strychnine-insensitive Gly modulatory sites have been reported in normal aging and in Alzheimer's disease (24, 28, 33). Additionally, Gly itself has been shown to play a role in NMDA receptor-mediated excitotoxicity (26). In previous studies, we have found a moderate reduction in the [^{3}H]-glutamate binding to NMDA receptors in some brain areas of Mn-treated mice, probably due to a down-regulation of the expression of NMDA sites (8). Because of the close relationship between both recognition domains, the [^{3}H]-Gly binding to strychnine-insensitive Gly modulatory sites was analyzed by autoradiographic methods in the brain of Mn-treated mice, to determine whether these sites are altered in this neurotoxic disorder.

MATERIALS AND METHODS

Materials

[2-^{3}H]-Glycine (48.4 Ci/mmol) was obtained from NEN-Du Pont (Boston, MA). Manganese chloride ($MnCl_2.4H_2O$) was acquired from Fisher Scientific Co. (Fair Lawn, NJ). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO).

Tissue preparation

Male albino mice (NMRI-IVIC strain) were injected intraperitoneally with manganese chloride (5 mg Mn/kg body weight/day) during 8 weeks (5 days/week). Control animals were treated similarly with saline solution (0.025 M NaCl). Two days after the last injection, animals were anesthetized and perfused intracardially with cold 0.01% formaldehyde in 0.05 M phosphate buffered saline (pH 7.4). The brains were quickly removed and frozen on dry ice. They were stored at -80°C until use. Horizontal sections of 15 %m from both groups of animals were

cut on a Kryomat cryostat (-18°C) and thaw-mounted on acid washed and gelatin-subbed microscope slides.

Autoradiographic methods

Binding procedures were performed according to a previous described method with slight modifications (25). Briefly, slides were warmed to room temperature prior to incubation. Tissue sections were first preincubated in 50 mM Tris-citrate (pH 7.4) at 4°C for 30 min to remove endogenous compounds. Sections were then incubated in Tris-citrate buffer containing 100 nM [^{3}H]-Gly and 100 µM strychnine for 35 min at 4°C. The concentration of [^{3}H]-Gly used in this procedure represents approximately half of the Kd value (170-200 nM) (25). Non-specific binding was determined in the presence of 1 mM unlabeled Gly. After the incubation, sections were rinsed three times in ice-cold buffer followed by a final rinse with cold 2.5% glutaraldehyde in acetone and quickly blown dried with warm air to minimize dissociation. Tissue sections along with methacrylate standards (^{3}H-Microscales, Amersham) were apposed to tritium-sensitive film (Hyperfilm-^{3}H, Amersham) for 4 weeks at 4°C. After that time, films were developed in Kodak D-19 at 20°C and then fixed with Rapid Fix. Quantitative analysis of the resulting autoradiograms was performed densitometrically using a microcomputer image processing system (Imaging Research Inc., St. Catharines, Ontario). Twenty or more readings from each area of interest were averaged, and the radioactivity values were converted to fmol/mg of tissue using a computer-generated polynomial regression analysis, which compared film densities produced by the tissue sections to those of radioactive standards. Brain regions were identified by overlapping of autoradiograms and cresyl violet-stained tissue sections.

Manganese determinations

Animals were sacrificed by cervical dislocation and the samples were taken from unfixed brains, using metal-free material. External contamination with Mn was avoided by rinsing the samples with doubly distilled demineralized water to reduce the error from blood contamination (4).

Brain Mn content was determined by flameless atomic absorption spectrophotometry, using a Perkin-Elmer model 2380 AAS with an HGA-2100 graphite furnace (4). All samples were analyzed by the method of standard additions.

Data analysis

Data from regional distribution of [^{3}H]-Gly binding in both groups were compared statistically using Student's independent t-test. Significance was assumed to be at $p < 0.05$. All density data were expressed in fmol/mg of tissue (wet weight).

RESULTS

Mn concentration increased significantly in the brain of treated animals. The means S.E.M. of Mn

content (n=7), expressed in μg/g of dry weight, were: striatum, 2.45 ± 0.53 in control and 6.60 ± 0.47 in treated mice; frontal cortex, 2.43 ± 0.52 and 5.50 ± 0.44; and hippocampus, 2.07 ± 0.39 and 6.20 ± 0.45, respectively.

The distribution of [^{3}H]-Gly binding to strychnine-insensitive Gly recognition sites in control mouse brain sections exhibited a marked heterogeneity, with the following rank order of binding densities: hippocampal formation cerebral cortex basal ganglia cerebellum. This distribution is in agreement with previous reports (25, 28). The regional distribution of [^{3}H]-Gly binding sites for control and Mn-treated animals is summarized in Table I. Twenty brain regions were analyzed densitometrically, and among all these regions only the globus pallidus exhibited a statistically significant decrease in [^{3}H]-Gly binding. The pars anterior and the pars posterior of the globus pallidus presented a 27% and a 28% reduction respectively.

DISCUSSION

In the present study, we have analyzed the distribution of strychnine-insensitive Gly binding sites in the brain of Mn-treated mice, using autoradiographic binding techniques. As a result, the globus pallidus was the only area affected by a [^{3}H]-Gly binding decrease, while the rest of analyzed areas did not show any significant change. The globus pallidus exerts an inhibitory control on the subthalamic nucleus, which in turn sends a less-dense excitatory glutamatergic projection to the globus pallidus (22). Therefore, the pallidum-subthalamic nucleus system establishes a closed loop within the basal ganglia (22). Accumulated pathological evidence indicates that the pallidum-subthalamic nucleus system may be preferentially damaged in Mn encephalopathy in both humans and animals. In fact, the first proper description of human brain changes in Mn poisoning particularly emphasizes the pallidal degeneration (1). Further studies reported evident lesions mainly in the pallidum, caudate nucleus and putamen (3, 7, 15, 30, 34, 36). Yamada and col. also reported an autopsy case with degeneration of the basal ganglia, in which the pallidum was severely affected (38). Animal models of Mn intoxication confirms these observations. Therefore, severe lesions in the pallidum and subthalamic nucleus of monkeys treated with Mn dioxide have been observed, and these lesions were identical to those reported in 3 cases of human Mn poisoning, which involved primarily the pallidum (31, 32). Eriksson and col. observed a 50% loss of dopamine from globus pallidus in monkeys treated subcutaneously with Mn oxide (14). Additionally, magnetic resonance images in intravenously Mn-treated monkeys showed that this metal accumulates initially in the globus pallidus, reaching the highest levels in the central nervous system, and

TABLE I
REGIONAL DISTRIBUTION OF STRYCHNINE-INSENSITIVE [^{3}H]-GLYCINE BINDING SITES IN THE BRAIN OF MANGANESE-TREATED MICE

AREA	CONTROL	TREATED
Cortex:		
Orbital	109.9 ± 8.8	106.7 ± 9.4
Cingulate	148.4 ± 15.5	140.2 ± 12.6
Frontal	101.3 ± 6.2	93.5 ± 9.7
Parietal	94.3 ± 5.4	99.1 ± 10.6
Temporal	114.1 ± 8.5	116.8 ± 10.5
Entorhinal	120.6 ± 4.8	109.8 ± 12.1
Basal Ganglia:		
Lateral Septum	108.3 ± 5.6	94.4 ± 10.2
Globus pallidus (ant)	57.4 ± 4.9	41.9 ± 5.4*
Globus pallidus (post)	50.5 ± 3.3	36.5 ± 3.2*
Caudate-putamen (ant)	89.3 ± 8.5	82.4 ± 7.5
Caudate-putamen (post)	80.9 ± 4.3	75.3 ± 7.2
Caudate-putamen (lat)	80.2 ± 8.4	76.7 ± 9.1
Caudate-putamen (med)	78.9 ± 10.8	60.7 ± 9.8
Hippocampus:		
Dentate gyrus	169.8 ± 15.1	141.2 ± 13.6
Subiculum	122.4 ± 9.9	110.7 ± 7.2
Area CA1	181.5 ± 10.2	152.9 ± 12.1
Area CA2	204.1 ± 12.3	173.8 ± 9.7
Area CA3	167.8 ± 13.5	140.2 ± 11.6
Cerebellum:		
Gran. and mol. layers	69.2 ± 8.2	62.3 ± 6.5
White matter	38.6 ± 5.1	29.7 ± 4.7

Values represent the mean ± S.D. from 4 control and 4 treated mice. These are expressed in fmol/mg of tissue. *Significance was assumed to be at $p<0.05$.

later it is redistributed to other regions (29).

Taking together, these evidences support the hypothesis that during Mn poisoning the decrease in strychnine-insensitive [^{3}H]-Gly binding observed by us, restricted to the globus pallidus, could probably reflect a degeneration of structures containing Gly binding sites. However, a possible regulation of Gly site expression can not be ruled out since Gly sites might be in neurons resistent to Mn-induced degeneration. Thus, a down-regulation of Gly binding sites without changes in other recognition sites of the NMDA receptor complex in the globus pallidus might be plausible, since changes in the stoichiometry of the complex have been previously reported in aging rats (24, 28) and in neuropsychiatric disturbances (35). In addition, the numerical relation between the principal recognition sites that comprise the NMDA complex is highly variable among brain regions (25).

In a previous report we found a significant reduction in [^{3}H]-glutamate binding to NMDA sites, primarily in all areas of the hippocampal formation (15-21%) and in the basal ganglia (10-16%), except the globus pallidus, which was unaltered (8). Although Glu and strychnine-insensitive Gly binding sites are colocalized on the NMDA complex, surprisingly during Mn poisoning Gly and NMDA binding site reductions do not coincide in the same structures. Gly binding sites are decreased only in the globus pallidus whereas NMDA sites appear diminished in the majority of analyzed areas, except the globus pallidus.

We proposed that the decrease in NMDA binding sites during Mn poisoning could be due to a down-regulation of NMDA receptors by excessive activation of Glu binding sites in the NMDA receptor complex. Since this down-regulation was moderate and diffuse, and affects the majority of analyzed areas, it might be considered as a protective mechanism against a delayed excitotoxic process. It is important to note that an excessive activation of glutamatergic receptors has been reported sequentially associated to oxidative stress (11), which is the primary degenerative mechanism occurring in Mn intoxication (2, 6, 12, 17, 20).

Because of its complexity, the NMDA receptor provides several possible targets for compensatory control mechanisms, including the Glu/NMDA binding site, the ion channel, and the Gly modulatory site (21), which makes unnecessary the regulation of the entire NMDA receptor complex. In this regard, agonist occupation of both Glu and Gly recognition sites is a minimal requirement for receptor activation (19), and therefore changes in one of the recognition sites would be enough to regulate NMDA receptor activation.

A possible explanation for the mismatch found between Gly and NMDA binding site reductions might be an up-regulation of NMDA bind-

ing sites in the globus pallidus to compensate the effect of neuronal degeneration in this region. Since the globus pallidus has very low excitatory innervation (25) and most of the basal ganglia circuities are inhibitory (22), it is necessary to secure a level of transmitter-dependent activity necessary to maintain synaptic stability (21). Moreover, the secondary effects of a blockade or an accentuated reduction of NMDA receptors could be very noxious (16).

In this context, the regulation of the NMDA binding site (up and down) could be the principal compensatory mechanism of the NMDA complex to control the levels of synaptic activity in order to avoid either excitotoxicity or hipoactivity.

Finally, it might not be ruled out the possibility that some strychnine-insensitive Gly sites may not be associated with the NMDA complex. In fact, it is not clear whether these binding sites are always related or if they can exist independently of one another (25). Therefore, changes in these putative Gly sites should not affect the NMDA receptor complex.

Whatever mechanism is responsible for the Gly binding decrease in the globus pallidus, this structure, like the striatum, plays a basic role in motor behavior, particularly in the automatic execution of highly learned motor patterns, as well as in higher-order aspects of cognition and behavior (22). Therefore, this alteration of Gly binding sites in the globus pallidus could be involved in the alterations of both motor and behavioral patterns reported during Mn intoxication (5, 9).

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