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Copper sulfate prevents tyrosine hydroxylase reduced activity and motor deficits in a Parkinson's disease model in mice

Mireya Alcaraz-Zubeldia,* Marie Catherine Boll-Woehrlen,* Sergio Montes-López,* Francisca Pérez-Severiano,* Juan Carlos Martínez-Lazcano,* Araceli Díaz-Ruiz,* Camilo Ríos*

* Departamento de Neuroquímica. Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suárez.

ABSTRACT

Introduction. Parkinson's disease (PD) is a neurodegenerative disorder characterized by the presence of motor disturbances, derived from the striatal dopamine depletion. Previously, we reported that $CuSO_4$ pretreatment blocked an oxidative stress marker (lipid peroxidation) and prevented the striatal dopamine depletion induced by the administration of the 1-methyl-4-phenylpiridinium (MPP+), the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a model of PD. **Objective.** To determine if tyrosine hydroxylase (TH), the rate-limiting synthetic enzyme of dopamine, is implicated in the neuroprotective effect of $CuSO_4$ pretreatment, and if this neuroprotective effect is able to prevent the hypokinetic state (measured as spontaneous locomotor activity, SLA) induced by the experimental model of PD. **Material and methods.** C57 Black/6J mice received a single dose of $CuSO_4$ (2.5 mg/kg, i.p.) either 16 or 24 h before the administration of MPP+ (18 µg/3 µl, i.c.v.). Twenty four hours later, mice SLA was registered and animals sacrificed. Striatal L-DOPA accumulation derived from the administration of a central dopamine descarboxilase inhibitor was evaluated, a strategy considered as a reliable indirect analysis of tyrosine hydroxylase activity (THA). **Results.** Administration of MPP+ decreased SLA (-52%; $p = 0.003$) as compared to control group values, whereas those mice pretreated with $CuSO_4$ 16 h before MPP+, increased SLA by 47% as compared with control group ($p = 0.015$). Mice pretreated with $CuSO_4$ 24 h before MPP+, also showed a statistically significant increase in SLA (71%; $p = 0.02$), when compared with control group. As a consequence of MPP+ administration, THA was also reduced as compared to control group values (32%; $p < 0.05$). Reduction of THA was blocked when mice were pretreated with $CuSO_4$ 16 h before MPP+. Moreover, mice receiving the $CuSO_4$ 24 h before MPP+ showed a significant increase (38%; $p < 0.05$) in THA when compared with control group. **Con-**

El sulfato de cobre previene del deterioro en la actividad de la tirosina hidroxilasa y de la rigidez inducidas por un modelo de la enfermedad de Parkinson en ratones

RESUMEN

Introducción. *La enfermedad de Parkinson (EP) se caracteriza por trastornos motores derivados de la disminución en el contenido de dopamina estriatal. En estudios previos el pretratamiento con $CuSO_4$ evitó el estrés oxidante (peroxidación de lípidos) y previno la disminución en el contenido de dopamina estriatal, eventos que caracterizan a la EP, inducidos por la administración del 1-metil 4-fenilpiridinio (MPP+), metabolito activo de la neurotoxina 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP), un modelo experimental de la EP.* ***Objetivo.*** *Comprobar si la neuroprotección ejercida por el $CuSO_4$ involucra mecanismos de compensación funcional dopaminérgica (THA), capaces de prevenir la rigidez inducida por el MPP+.* ***Material y métodos.*** *Ratones C57 Black/6J recibieron el $CuSO_4$ (2.5 mg/kg, i.p.) 24 o 16 h antes de la aplicación del MPP+ (18 µg/3 µl, i.c.v.). Después de 24 h se registró la actividad locomotriz espontánea (SLA) de los animales y fue analizada la acumulación estriatal de L-DOPA, provocada por la administración de un inhibidor central de la dopamina-descarboxilasa, considerado como un buen método indirecto para determinar la actividad de la tirosina hidroxilasa (THA).* ***Resultados.*** *La administración del MPP+ provocó hipocinesia en los animales (-52%; $p = 0.003$). La administración del $CuSO_4$ 16 h antes del MPP+ mostró un incremento de 47% ($p = 0.015$) en la SLA, mientras que ésta fue de 71% ($p = 0.02$) al ser administrado 24 h antes del MPP+, en comparación con el grupo control (tratado con Na_2SO_4). Asimismo, el MPP+ provocó una disminución estadísticamente significativa (-32%; $p < 0.05$) en la THA comparándola con los valores control. Dicha disminución se previno con el pretratamiento*

clusion. Results suggest that preservation of THA participates in the neuroprotective effects derived from the copper supplementation, a phenomenon that avoid the hypokinetic state induced by the MPP+ experimental model of PD.

Key words. Copper. Tyrosine hydroxylase. 1-methyl-4-phenylpiridinium. Locomotor activity. Parkinson.

de $CuSO_4$ 16 h antes de la administración del MPP+, mientras que aquellos ratones inyectados 24 h antes mostraron un incremento significativo (38%; $p < 0.05$) con respecto al control. ***Conclusión.*** *Estos resultados demuestran la participación de la tirosina hidroxilasa en la neuroprotección derivada del suplemento de cobre, lo que previno la rigidez inducida por el MPP+, modelo experimental de la EP.*

Palabras clave. *Cobre. Tirosina hidroxilasa. 1-metil-4-fenilpiridinio. Ratones. Actividad locomotriz espontánea. Parkinson.*

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the accelerated death of dopaminergic neurons located in the *substantia nigra pars compacta* (SNc), causing depletion of striatal dopamine (DA) content. Oxidative stress has been implicated in the neurodegenerative processes involved in the loss of SNc neurons and striatal DA depletion in PD.[1,2] When the homeostatic balance between free radicals release and the antioxidant defensive system fails, oxidative stress establishment causes serious damage to DNA, proteins, and membrane lipids.[3] Since copper acts as cofactor of several antioxidant defenses, its impaired transport and/or absorption could predispose individuals to oxidative stress.[4] Moreover, copper has been related with PD, since pathological conditions such as aceruloplasminemia, characterized by a lack of its main transport protein in plasma (ceruloplasmin) are linked to the appearance of biochemical and motor disturbances similar to those seen in PD (increased lipid peroxidation, basal ganglia degeneration and extrapyramidal abnormal movements).[5]

Previously, we have reported the ability of $CuSO_4$ to prevent oxidative stress (by blocking the increased rate of lipid peroxidation [LPO] and the striatal DA depletion, induced by the intracerebroventricular administration of 1-methyl-4-phenylpiridinium iodide (MPP+; 18 µg/3 µl, i.c.v.), a murine model of Parkinson's disease. This model is able to resemble the main biochemical and motor disturbances of PD, including the inhibition of the mitochondrial complex I, the increased accumulation of iron in basal ganglia, the dopamine and copper striatal depletion, the increased lipid peroxidation levels and the rigidity, all features found in PD patients. Our previous studies showed that both striatal DA depletion and increased LPO were completely blocked in those mice pretreated with $CuSO_4$ (2.5 mg/kg, i.p.), 24 h before the MPP+ injection.[6]

Regarding oxidative stress markers, the mitochondrial isoform of superoxide dismutase (Mn-SOD), but not the cytosolic isoform (Cu/ZnSOD) has been reported to be raised in PD brain tissues obtained from *post mortem* studies.[7] Nowadays, complex I dysfunction is considered an early biochemical feature of PD.[8] Previous results of our group corroborated that MnSOD is selectively activated by the MPP+ administration to mice.[9] The differential response elicited by MnSOD could be related with its ability to inactivate the superoxide radicals released as a consequence of the exposure the mitochondrial complex I inhibitor, the MPP+.[10]

On the other hand, Tyrosine hydroxylase (TH), the dopamine rate limiting enzyme, is regulated by several mechanisms.[11] TH by-product inhibition (L-dihydroxyphenylalanine; L-DOPA) respond to the high affinity of catecholamines (CATs) by interfering with the iron located at its catalytic domain.[12] CATs form coordinated bonds with this Fe^{+3} site, rendering TH unable to participate in the dopamine synthesis. In addition to CATs, nitric oxide also inactivates TH through its interaction with the Fe(III) site, or by the direct nitration of the tyrosine residues of TH by peroxynitrite.[13] Whereas TH immunoreactivity (TH-IR) is considered as the best immunochemical marker of dopaminergic neurons,[14] rigidity is frequently related to both the striatal dopamine content and TH activity (THA).[15]

In this study, THA was assessed *ex vivo* by measuring the accumulation of striatal L-DOPA 30 min after the systemic administration of a centrally active inhibitor of DOPA decarboxylase, 3-hydroxybenzyl hydrazine (NSD 1015, 100 mg:kg). The aim of this study was to determine if TH activity (THA) and the spontaneous locomotor activity (SLA), endpoints directly related with dopaminergic nigrostriatal integrity, could be preserved by $CuSO_4$ pretreatment in mice exposed to the Parkinson's disease model based on the MPP+ i.c.v. administration.

MATERIAL AND METHODS

All experiments were conducted under approval of the institutional animal welfare committee. Male C57 Black/6J adult mice (n = 8-10 per group), weighing 25-30 g, were used. Animals were fed *ad libitum* with standard rodent Purina Chow. Mice were randomly allocated into either one of six groups and administered with a single intraperitoneal (i.p.) dose of either $CuSO_4$ (2.5 mg/kg) or equimolar Na_2SO_4 solution. Dopaminergic damage was experimentally induced by the intracerebroventricular (i.c.v.) administration of 1-methyl-4-phenylpyridinium (MPP+ iodine, Sigma-Aldrich Co. St. Louis MO, USA; 18 µg/3 µl) or saline solution, according to previous reports[6] at either 16 h or 24 h after the $CuSO_4$ or Na_2SO_4 pretreatment. Spontaneous locomotor activity (SLA) was assessed 24 h after the MPP+ administration by an automated device (Opto-Varimex Minor equipment, Columbus Instruments; Columbus Ohio, USA) consisting of cages (45 x 45 x 15 cm) placed within two series of 15 infrared beams projected from either vertical or horizontal photocells associated with their corresponding sensors. Irruption of each infrared beam was registered and defined as either a horizontal, vertical or ambulatory movement. Locomotion occurred when the mouse moved horizontally through the low level grid of infrared beams. Since neither ambulatory nor vertical movements showed statistically significant differences compared to the control group, the results derived from locomotion are defined as spontaneous locomotor activity (SLA). The animals were evaluated only once between 8:00-13:00 h, under the same environmental conditions of light intensity and temperature. Experimental groups were tested in a counterbalanced order. Mice were kept inside the cage for free movement habituation during 30 min and SLA was assessed for one hour, based on previous reports.[16] Results are expressed as the number of counts registered *per* hour.

Twenty four hours after SLA measurement, mice received the administration of the 3-hydroxybenzyl hydrazine (NSD 1015; 100 mg/kg, i.p.; Sigma-Aldrich St. Louis MO, USA), a L-DOPA decarboxylase inhibitor (DDC), in order to fully inhibit the L-DOPA descarboxylation, and with the aim of analyzing the striatal *ex vivo* tyrosine hydroxylase activity (THA) by measuring the accumulation of L-DOPA, according to previous reports.[17] Thirty minutes after the administration of the centrally active NSD 1015, mice were killed by decapitation, and their brains were quickly removed; the striatum was then dissected out on ice as described by Glowinski and Iversen[18] and carefully weighed. Five-hundred-µl of a perchloric acid/ sodium metabisulfite solution (0.1% w/v) was added to the tissue and homogenized. Samples were then centrifuged at 4,000 x g for 10 min, supernatants covered from light and kept at -70 ºC until analyzed. Striatal content of L-DOPA was analyzed using a High Performance Liquid Chromatography (HPLC) system (LC 250 Perkin Elmer) with a Metrohm 641-electrochemical detector[6] and catecholamine analytical column (Adsorbosphere, 4.5 x 100 mm, 3 µm particle size, Alltech Associates, Inc Deerfield, IL). In order to optimize L-DOPA analysis, mobile phase was modified from previous reports[19] with phosphate buffer (pH = 2.55) containing 0.2 mM sodium octyl sulfate, 0.1 mM EDTA, and 16.5% (v/v) of methanol. Signal Peaks were integrated using a Turbochrom 4.0 Software (Perkin Elmer). Results of the samples were interpolated in calibration curves previously constructed for L-DOPA. THA was expressed as µg of accumulated L-DOPA per gram of wet tissue/hour (mean ± SEM).

Catalytic activity of constitutive Ca^{+2}-dependent NOS (including, eNOS and nNOS isoforms), as well as the inducible Ca^{+2}-independent NOS (iNOS isoform) was determined by the conversion of [H^3]-L-arginine to [H^3]-L-citrulline according to previous reports.[20] Striatal tissue was homogenized in 250 µl of buffer (50 mM Tris–HCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol, pH 7.5) containing a cocktail of protease inhibitors (100 µM leupeptin, 1 mM phenylmethylsulphonyl fluoride, 2 µg/mL aprotinin, 10 µg/mL soybean trypsin inhibitor and 0.1% v/v Nonidet NP-40). A volume of homogenized solution containing 500 µg of protein was incubated for 30 min at 37 ºC in the presence of 10 µM L-arginine-HCl, 0.2 µCi [3H]-L-arginine, 1 mM NADPH, 100 nM calmodulin, 2.5 mM $CaCl_2$, and 30 µM BH_4. To assay the activity of Ca^{+2}-independent iNOS, the incubation was performed in the presence of 0.1 mM EGTA and 0.1 mM EDTA with no $CaCl_2$. Reactions were stopped by adding a buffer containing 2 mM EGTA, 2 mM EDTA and 20 mM HEPES, pH 5.5. The reaction mixture was applied onto a 1 mL column of cation interchange resin (Dowex-50W), which had been previously equilibrated with stop buffer. This column retains labeled arginine and allows [H^3]-L-citrulline to elute through the column. [H^3]-L-citrulline was quantified using a Beckman LS6500 scintillation counter. Results were expressed as ng [H^3]-L-citrulline/500 µg of protein *per* 30 min.

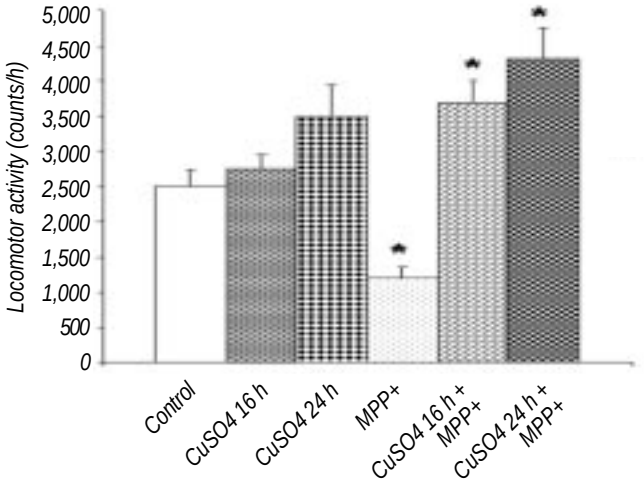

Figure 1. *Spontaneous Locomotor Activity (SLA). SLA is represented as the number of counts in 30 minutes. Results are expressed as the mean ± S.E.M. of n = 8-10 independent experiments. Data obtained were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney multiple comparisons' test. SPSS version 10.0 Software was employed for statistical analyses. Statistically significant values compared to control group *p < 0.02. Control: Mice pretreated with Na_2SO_4 (2.5 mg/kg; i.p.) 24 h before the intracerebro-ventricular administration (i.c.v.) of saline solution. $CuSO_4$ 16 h and $CuSO_4$ 24 h: Mice pretreated with copper 16 h or 24 h before the administration of saline solution (0.9%; i.c.v.), respectively. MPP+: Mice pretreated with Na_2SO_4 (2.5 mg/kg, i.p.) 24 h before the MPP+ administration (18 µg/3 µl; i.c.v.). $CuSO_4$ 16 h + MPP+ and $CuSO_4$ 24h + MPP+: Mice pretreated with $CuSO_4$ (2.5 mg/kg, i.p.) either at 16 h or 24 h before the MPP+ injection, respectively.*

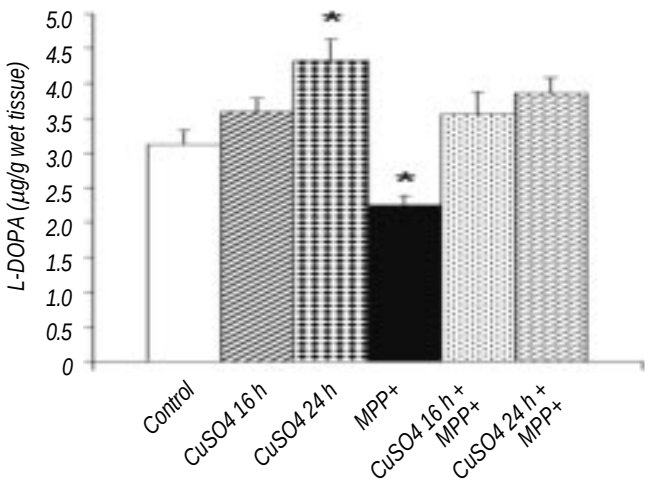

Figure 2. *Tyrosine hydroxylase activity (THA). Indirect analysis of THA was performed by evaluation of striatal L-DOPA content in presence of the dopa-descarboxilase inhibitor NSD-1015 (100 mg/kg, i.p.). Results are expressed as the mean ± S.E.M. of n = 8-10 independent experiments. Data derived from L-DOPA accumulation (THA) were analyzed using the two-way ANOVA followed by Tukey's test with the SPSS version 10.0 Software. Statistically significant values compared to control group *p < 0.05. Control: Mice pretreated with Na_2SO_4 (2.5 mg/kg; i.p.) 24 h before the intracerebro-ventricular administration (i.c.v.) of saline solution. $CuSO_4$ 16 h and $CuSO_4$ 24 h: Mice pretreated with copper 16 h or 24 h before the administration of saline solution (0.9%; i.c.v.), respectively. MPP+: Mice pretreated with Na_2SO_4 (2.5 mg/kg, i.p.) 24 h before the MPP+ administration (18 µg/3 µl; i.c.v.). $CuSO_4$ 16h + MPP+ and $CuSO_4$ 24h + MPP+: Mice pretreated with $CuSO_4$ (2.5 mg/kg, i.p.) either at 16 h or 24 h before the MPP+ injection, respectively.*

Statistical analysis

Data derived from L-DOPA accumulation (THA) were analyzed using the two-way ANOVA followed by Tukey's test. Data obtained from spontaneous locomotor activity (SLA) tests were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney multiple comparisons' test. The SPSS version 10.0 Software was employed for statistical analyses. Values of $p < 0.05$ was considered of statistical significance.

RESULTS

Spontaneous Locomotor Activity (SLA)

Spontaneous Locomotor Activity (SLA) showed a 52% significant reduction if MPP+ was administered to mice (1,192.11 ± 161.68 counts/h), in comparison to control values (2,509 ± 233.86 counts/h; $p = 0.003$). On the contrary, those animals exposed to $CuSO_4$ in absence of MPP+, showed a non-significant increase of 9% on SLA (2,755 ± 196.83 Counts/h) and 39% (3,491.5 ± 447.39 counts/h) when copper was injected 16 h or 24 h before the intracerebroventricular administration of saline solution, respectively. On the other hand, $CuSO_4$ pretreatment blocked the motor deficit induced by MPP+ in mice. A 47% statistically significant increase (3,688.33 ± 305.88 counts/h; $p = 0.015$) was observed in SLA at 16h, whereas a 71% statistically significant increase (4,309.75 ± 437.91; $p = 0.02$) in SLA was observed when the $CuSO_4$ pretreatment was given 24 h before MPP+, as compared to control group values (Figure 1).

Tyrosine Hydroxylase Activity (THA)

Mice treated with MPP+ showed a significant 28% decrease in the THA (2.24 ± 0.14 µg/g/h of L-DOPA, $p < 0.05$) when compared with control group values (3.12 ± 0.19 µg/g/h of L-DOPA). Meanwhile, the group receiving the copper sulfate-only treatment, showed an increased activation of TH of 14% (3.58 ± 0.19 µg/g/h of L-DOPA) and 38% (4.3 ± 0.31 µg/g/h of L-DOPA) at 16 h or 24 h, respectively

when compared to control group, and being statistically significant this difference only in those animals injected 24 h before sacrifice ($p < 0.05$; Figure 2). In addition, pretreatment with $CuSO_4$ successfully blocked the TH inactivation produced by MPP+, when administered both at 16 h (3.56 ± 0.30 µg/g/h of L-DOPA) or 24h (3.84 ± 0.23 µg/g/h of L-DOPA). Both results did not differ from control group values.

NOS activity assay

Results obtained from the activity of the constitutive Ca^{2+}-dependent NOS (eNOS and nNOS isoforms) as well as the inducible Ca^{2+}-independent NOS (iNOS) showed that $CuSO_4$ pretreatment was unable to modify the activity of none of the NOS isoforms *ex vivo* (data not shown).

DISCUSSION

Motor disturbances have been reported for both, humans[21] and mice[22] as a consequence of either the dopamine depletion or diminished tyrosine hydroxylase activity (THA). In agreement to previous reports, we found that the MPP+ administration induced a marked decrease of both the THA and the spontaneous locomotor activity (SLA) in mice.[23,24] Such a decrease could be partially explained by the ability of MPP+ to promote the quinotyrosine production,[25] and/or by nitration of the tyrosine residues of tyrosine hydroxylase.[26] This quinotyrosine production could be, in turn, a consequence of direct interaction of MPP+ with CATs, or by its ability to favor the overproduction of reactive oxygen species through inactivation of mitochondrial complex I by MPP+.[27] In this regard, $CuSO_4$ pretreatment protected rodents from oxidative stress and glucose overload in an experimental model of diabetes.[28] Nitric oxide (NO) overproduction favors the formation of peroxynitrite[29] and several studies have demonstrated the participation of nitric oxide in the neurotoxicity exerted by MPTP/MPP+.[10,23] There is evidence that the inhibition of the neuronal nitric oxide synthase (nNOS) provides neuroprotection from the MPTP neurotoxin,[30] and that this isoform could be inhibited by copper ions.[31,32] Since this mechanism has not been tested to occur *in vivo*, we evaluated the nitrergic system *status* through the analysis of the activity of nitric oxide synthase constitutive isoforms. Results from the present study suggest that $CuSO_4$ pretreatment did not modify the activity of NOS isoforms *in vivo* (data not shown). Thus, it is less likely that this mechanism is involved in the protection exerted by copper sulfate.

On the other hand, ceruloplasmin synthesis responds to copper availability. Thus, $CuSO_4$ pretreatment could enhance ceruloplasmin expression, which in turn could protect dopaminergic neurons by scavenging superoxide radicals, but also by preventing peroxynitrite synthesis by diminishing the availability of nitric oxide through its ability as a nitrite synthase.[32]

Previously, we demonstrated that $CuSO_4$ pretreatment blocked the striatal dopamine depletion induced by MPP+. Pretreatment with $CuSO_4$ could participate in the protection against the striatal dopamine depletion induced by MPP+, through the activation of tyrosine hydroxylase observed when copper was administered at both the 16 and 24 h previous to the experimental model of PD. Dopaminergic status could be also influenced by the role of copper as cofactor of several enzymes involved in the dopamine metabolism, which could increase the dopaminergic tone. This suggestion is supported by the hyper-activation of tyrosine hydroxylase in those mice exposed to $CuSO_4$ in absence of MPP+, as compared with control values.

Copper neuroprotective effects could be related to the activation of several antioxidant defenses.[19] Moreover, both transgenic mice overexpressing the copper/zinc superoxide dismutase (Cu/Zn-SOD) and those receiving superoxide dismutase mimetic agents are protected from MPTP neurotoxicity.[7,33] Our previous findings demonstrated that $CuSO_4$ exerts neuroprotection against oxidative stress in those mice exposed to MPP+,[6] and that this neuroprotective effect could be partially explained by the activation MnSOD.[9] Nevertheless, other copper-related antioxidant enzymes like metallothionein and ceruloplasmin should not be discarded to participate in this neuroprotection.[1]

Our results employing the intracerebroventricular administration of MPP+ to mice (active metabolite of MPTP) instead of the MPTP neurotoxin, corroborate the reduced spontaneous locomotor activity (SLA), previously reported for MPTP.[17] Copper exposure has been previously linked with increased motor behavior. Moreover, the increased SLA induced by $CuSO_4$ pretreatment to mice could be related with an increased dopamine response. This suggestion is supported by previous reports obtained from dietary supplement of copper. Animals subjected to a copper-rich diet, showed an increased motor activity associated directly with dopamine increased levels.[34] Nevertheless, results obtained from

the co-administration of $CuSO_4$ and MPP+, constitute the first report of the copper protective potential against the reduced SLA induced by MPP+. There is evidence that caffeine and more specific antagonists of the adenosine A_{2A} receptor protect dopaminergic neurons from the MPTP Parkinson's disease model[35] and stimulate locomotor behavior.[36]

The neuroprotective effects of $CuSO_4$ against the motor disturbances induced by MPP+ could be related with the ability of copper to irreversibly inhibit the binding of adenosine agonists (A_{2A} receptors).[36] These results, in addition to our previously reported neuroprotection exerted by the $CuSO_4$ pretreatment against the striatal dopamine depletion induced by MPP+,[6] suggest the participation of an adenosine-related effect over the dopaminergic system triggered by the $CuSO_4$ administration. Adenosine receptor antagonism induced by copper could prevent both, the SLA deficits and the reduced activity of TH. Mechanisms related to THA[37] and SLA improvements induced by $CuSO_4$, must be further explored in order to explain the possible mechanisms of neuroprotection by $CuSO_4$ in the MPP+ model of Parkinson's disease.

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Correspondence and reprint request:

Dr. Camilo Ríos
Departamento de Neuroquímica
Instituto Nacional de Neurología y Neurocirugía
Manuel Velasco Suárez
Av. Insurgentes Sur No. 3877,
Col. La Fama, Tlalpan
14269, México, D.F.
Tel.: (55) 5606-3822, Ext. 2006
Fax: (55) 5424-0808
E-mail: crios@correo.xoc.uam.mx

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