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Supersensitivity of the cholinergic muscarinic system in the rat's brain is induced by high concentrations of Cu^{+2}.

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Key words: Muscarinic receptor, Cu^{2+}, locomotor activity, memory, super-sensitivity, [^{3}H]-QNB binding.

Abstract. Transition metals have been described as regulators of receptor's function. Here, we studied the effects of chronic administration of Cu^{2+} or the Cu^{2+} chelator penicillamine (PA) on the functional and binding properties of the muscarinic receptors (MR) on selected areas of rat's brain. Groups of 10 Sprague-Dawley rats were treated daily, for 45 days with either 1) 1mg/Kg $CuSO_4$ (Cu^{2+}), 2) 100 mg/Kg PA, or 3) saline solution. Double T-maze and motility cages were used for behavioral testing and the binding assays were performed using [^{3}H]-QNB or [^{3}H]-N-MSCP as MR's ligands. Cu^{2+} brain levels were measured in the cerebral cortex by atomic absorption spectrophotometer. Results showed that PA treated rats displayed a significant decrease of locomotor's activity (LA) and rearing behavior (RB), but a significant increases in memory efficiency (ME). Cu^{2+} treated rats displayed diminished RB with no significant changes in LA. Cu^{2+} treated rats displayed higher MR's density (Bmax) in cortex (C), striatum (S), and hippocampus (H). An increase in Bmax was also observed in PA treated rats, but only in C and S. Finally, Cu^{2+} tissue concentration was significantly higher in C of both Cu^{2+} and with PA treated animals. In conclusion, 45 days of Cu^{2+} or PA treatment induced brain hypercuprosis, which was associated with MR binding supersensitivity; however, change in ME was only observed in PA treated rats suggesting that might be still another factor in these experiments besides Cu^{2+} (i.e., Zn^{2+} or PA itself) involved in memory modulation.

Supersensibilidad colinérgica muscarínica inducida por altos niveles de Cu^{2+} en el cerebro de ratas.

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Palabras claves: Receptor muscarínico, Cu^{2+}, actividad locomotora, memoria, supersensibilidad, unión de [^{3}H]-QNB.

Resumen. Los metales de transición han sido descritos como reguladores de la función de los neurotransmisores. En este trabajo, nosotros estudiamos el efecto de la administración crónica de Cu^{2+} y del quelante de cobre penicilamina (PA) sobre las propiedades funcionales y bioquímicas del sistema colinérgico muscarínico. Tres grupos de ratas Sprague Dawley, de 10 individuos cada uno, fueron tratados con: 1) $CuSO_4$ (Cu^{2+}) (1 mg/kg); 2) PA (100 mg/kg); 3) solución salina fisiológica; diariamente, por 45 días. Cajas de motilidad y un laberinto doble T fueron utilizados para los experimentos conductuales y [^{3}H]-QNB o [^{3}H]-N-MSCP fueron utilizados como marcadores en los experimentos de unión de radioligandos. Los niveles de Cu^{2+} fueron medidos en corteza cerebral por espectrofotometría de absorción atómica. Los resultados mostraron que las ratas tratadas con PA mostraron una significante disminución en la actividad locomotora tanto vertical como horizontal de las ratas, así como un incremento significativo en la eficiencia de la memoria. Las ratas tratadas con Cu^{2+} solamente mostraron una disminución significativa en la actividad locomotora vertical. Estas ratas mostraron un aumento significativo de la densidad (Bmax) de receptores colinérgicos muscarínicos en la corteza cerebral, en el estriado y en el hipocampo. En las ratas tratadas con PA sólo se observó un incremento significativo en Bmax en la corteza cerebral y en el estriado. Finalmente, las concentraciones tisulares de Cu^{2+} en la corteza cerebral se encontraron significativamente aumentados, tanto en las ratas tratadas con Cu^{2+} como en las tratadas con penicilamina. En conclusión altos niveles tisulares de Cu^{2+} se asociaron con una supersensibilidad de los receptores colinérgico muscarínicos, sin embargo; el hecho de que un aumento en la eficiencia en la memoria fue solo observado en las ratas tratadas con PA sugiere que otros factores como por ejemplo Zn^{2+} y PA pudieron haber estado involucrados.

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INTRODUCTION

Muscarinic receptors (MRs) belong to the super family of seven transmembrane helix G-proteins-coupled receptors (1-3). Five MR subtypes: m1, m2, m3, m4, and m5 have been described based on molecular cloning studies; however, only four of these cloned subtypes have been pharmacologically and functionally defined, in primary tissues, corresponding to the described receptors M1, M2, M3, and M4 (2).

Cholinergic neurotransmission, besides its classical role in peripheral neurotransmission, is also involved in a variety of cerebral-controlled functions such as learn-

ing, memory, and cognition (4-6). Loss of cholinergic integrity, including low levels of brain's cholineacetyltransferase, loss of cortical cholinergic projections on the forebrain, and reduced number of MRs have been demonstrated in Alzheimer's disease, which is the most prevalent type of memory disorders (7). Furthermore, MR antagonists can disrupt acquisition and performance of learned behaviors by interfering with the interaction between acetylcholine and MRs (8).

Several endogenous factors have been reported to be able to modify functions of MR, among then, endogenous putative regulatory ligands (9,10), guanine nucleotides (11), and ions such as Cu^{2+} and Zn^{2+} (12).

Copper (Cu^{2+}) is an essential trace metal, which plays an important role in the biochemistry of human nervous system. Several studies have indicated that alterations in Cu^{2+} homeostasis is implicated in the pathogenesis of certain diseases characterized by neurodegenerative processes such as Menkes' and Wilson's diseases (both are inherited disorders of Cu^{2+} metabolism) (13) as well as in Alzheimer' disease, which is characterized by the imbalance of the Cu^{2+}, Fe, and Zn^{2+} homeostasis among other features (13, 14).

In vitro studies have shown that Cu^{2+} is able to affect MRs by inhibiting the muscarinic's antagonists binding to MR in hippocampus, forebrain, cortex, and adrenal medulla, but not to the brainstem's MRs (12, 15, 16). On the other hand, it has been reported that Cu^{2+} facilitates the binding of agonists by either decreasing the agonist's Ki (i.e., in displacement curves) or by increasing [^{3}H]-acetylcholine binding capacity in rat cortex (12, 16-19). The *in vivo* effect of Cu^{2+} has been explored only in rats with induced Cu^{2+}-deficincy, but the results are controversial. Farrar and Hoss (20) reported that homogenates prepared from forebrains of Cu^{2+}-deficient animals displayed a significant decrease in MR occupancy and affinity. Feller *et al.* (21) found that Cu^{2+} decreased MR occupancy only in corpus striatum and cerebral cortex however, Geiger *et al.* (22) reported the opposite phenomenon, with Cu^{2+} promoting increased [^{3}H]-QNB binding in striatum and cerebellum. Still, there are not studies addressing the *in vivo* effect of an excess of Cu^{2+} in the MRs' binding on experimental animals.

Regardless the cumulated knowledge related with the *in vitro* effect of Cu^{2+} on antagonist and agonist binding and the effect of Cu^{2+} deficiency on the expression of MR in rats, the role of Cu^{2+} on the MR-mediated aspects of behavior has not been clearly defined. For this reason, we decided to assess the effects of chronic administration of Cu^{2+} or the Cu^{2+} chelator penicillamine (PA) on learning, memory, and locomotor activities of young Sprague Dawley rats, followed by determination of MR binding affinity and occupancy in brain's homogenates, in order to correlate binding with behavioral findings. Our results showed that Cu^{2+} and/or PA both induced brain hypercuprosis and MR up-regulation. These molecular findings were translated in behavior changes (i.e., hypomotility and learning facilitation) suggestive of a functional supersensitivity of these receptors.

MATERIALS AND METHODS

Animals and treatments

3 groups, each composed of ten (n=10) 21-days old male Sprague-Dawley rats, received a daily intraperitoneal injection of: 1) 1 mg/kg $CuSO_4$ (Cu^{2+}), 2) 100 mg/kg PA, or 3) equal volume of 0.85% NaCl (control group) for 45 days. To avoid the PA's collateral effects, 2 mg/kg/day $ZnSO_4$ and 4 mg/kg/day pyridoxine were added to the drinking water. After treat-

ment, each animal from every group was evaluated for its learning capacity and locomotor activity. Then, the animals were sacrificed by decapitation and their brains rapidly removed. The striatum, brainstem, frontal cortex, and hippocampus were dissected. Frontal cortex and hipocampus were chosen because their involvement in memory processes and striatum because their involvement in motor funtions respectively; furthermore, frontal cortex and hippocampus, as well as striatum, are characteristically described to be rich in MR of the M1 subtype (6). The brainstem region was chosen because its high content of M2-subtypes MRs (2). The dissected regions from each group were cut in small pieces and homogenates prepared 1: 50 w/v in 25 mM Hepes Buffer pH 7.3, aliquoted in small volumes, and stored at –70°C until used. Protein concentration was determined by bicinchioninic acid colorimetric (BCA) assay (23).

Binding assays

[^{3}H]-QNB (an hydrophobic muscarinic ligand non subtype specific) and [^{3}H]-N--MSCP (an hydrophilic muscarinic ligand non subtype specific) were used to perform binding assays, according to protocols already published (12), in order to estimate the MR number in the dissected brain's regions. Briefly, 100 μg of total protein were incubated with 5-1000 pM [^{3}H]-QNB or 20-4000 pM of [^{3}H]-N-MSCP in absence (total binding) or presence (nonspecific binding) of 2 μM atropine in 2 mL final volume completed with 25mM Hepes buffer, at 37°C for 60 min. Binding reaction was terminated by vacuum filtration through GF/B glass-fiber filters (Whatman Inc. Clifton, NJ). Filters were washed three times with 5 mL of ice-cold phosphate buffer, dried (60°C for 12 hours) and placed into scintillation vials with 5 mL of scintillation cocktail (PPO, POPOP, Triton X-100, and Toluene). Radioactivity retained in the filters was measured in a liquid scintillation counter (57% counting efficiency; Wallac 1410, Pharmacia, Inc., Finland). Non-labeled atropine used in saturating concentrations blocks all muscarinic sites therefore, only non-muscarinic sites (nonspecific binding) are then available to the radioligands. In absence of atropine, all sites became accessible to the radioligand (total binding); therefore, specific (muscarinic) binding is obtained by subtracting nonspecific binding from the total binding values. Also, Bmax was determined at saturating concentrations of the ligand (500 pM of [^{3}H]-QNB).

In average, binding experiments were carried out in six independent points and repeated at least twice.

Behavioral testing

After 2 days of habituation trials, which consisted in let the animals freely explore the testing maze for 30 minutes (one trial per day), learning and memory were measured using a 20 seconds latency foot-shock double T Maze, based on a protocol described by Farr *et al.* (24). Memory was scored as the percent of successes and time that each rat expended in solving the maze in the trial (10 trials per test). Failures were defined as incapacity to solve the maze, enter in a wrong arm, or when receiving a foot-shock. Memory efficiency was defined as the percentage of success per unit of time. A motility cage, which is divided in four chambers, was used to score locomotor activity as well as the rearing behavior. Motility activity was recorded in 10 min periods preceded by a 5 min latency.

Behavior experiments were carried on each rat independently (n = 10 for each group).

Tissue's Cu^{2+} content determination

Cu^{2+} level in brain cortex was determined according to Eller and Haartz's protocol (25). Briefly, 150 mg of wet tissue

from each rat were digested in 1mL of 65% nitric acid at 70°C for 24 hours, diluted 1:4 v/v in deionized water, mixed and filtered trough Whattman 42 filter paper. Filtered solution was processed using an atomic absorption spectrophotometer (Pye Unicam SP 191) at 324.8 nm (air flow 5 L/min and acetylene flow 0.6 L/min). Results were expressed as μg of Cu^{2+} per gram of wet tissue.

Cortex Cu^{2+} content were determined on each rat independently (n = 10 for each group).

Data analysis

The maximum number of receptors (Bmax) and dissociation constant (Kd) were calculated based on the Hill's equation using a sigmoid curve from Graphpad Inplot Software (San Diego Ca.); adjustment was done according to the least square method. Data are expressed as the mean ± standard error. Statistical analysis of the differences between groups was performed using ANOVA test, followed by the Bonferroni post-test, accepting as significant a $p<0.05$.

Materials

[^{3}H]-QNB (l-quinuclidinyl[phenyl-4-^{3}H]-benzilate) with a specific activity of 48 Ci/mmol was purchased from Amersham Pharmacia Biotech UK and [^{3}H]-N-MSCP (scopolamine [N-methyl-^{3}H]-methyl chloride) with a specific activity of 80.4 Ci/mmol was obtained from New England Nuclear USA. All other reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

RESULTS

Binding characteristics of rat brain MRs

Brain's homogenates from Cu^{2+}-treated rats displayed a significant increase in MR density (Bmax) in cortex (2535 ± 41fmol/mg), striatum (1195 ± 30 fmol/mg), and hippocampus (2456 ± 26 fmol/mg) than control groups (2331 ± 39 fmol/mg in cortex, 1076 ± 9 fmol/mg is striatum, and 2309 ± 35 fmol/mg in hippocampus) as measured using [^{3}H]-QNB as radioligand. No significant changes in the number of binding sites in the brainstem between treated and control were observed. Unexpectedly, brain's homogenates of PA treated rats also shown a significant increase in the number of MRs in cortex (2555 ± 57 fmol/mg) and striatum (1248 fmol/mg); however, we did not detected significant changes in the number of MRs in hippocampus and brainstem (Table I). Similar results were observed using [^{3}H]-N--MSCP as radioligand (Fig. 1).

TABLE I
MUSCARINIC RECEPTOR DENSITIES IN DIFFERENT BRAIN AREAS

Structure	Treatments		
	Control	Cu^{2+}	PA
Cortex	2331 ± 39	2535 ± 41*	2555 ± 57*
Striatum	1076 ± 9	1195 ± 30*	1248 ± 31*
Hippocampus	2309 ± 35	2456 ± 26*	2340 ± 34
Brainstem	226 ± 12	227 ± 30	193 ± 10

Data are the mean ± standard error (n = 6 for each group).
Units are fentomols of [^{3}H]-QNB bound per mg of protein.
*$p < 0.05$ by ANOVA followed by Bonferroni post-test.

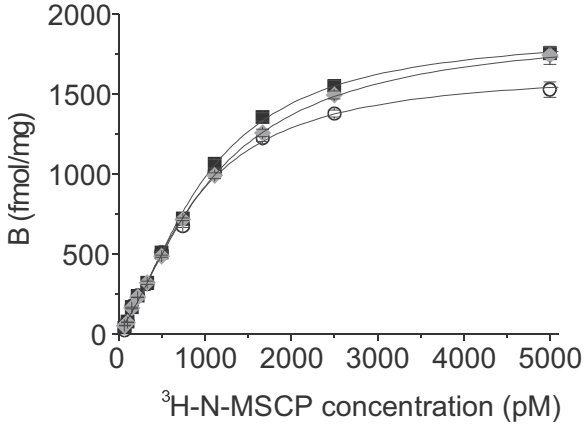

Fig. 1. Hyperbolic isotherm of [3H]-N-MSCP binding in cerebral cortex. 100 μg of protein from rat brain cortex were incubated with 20-4000 pM of [^{3}H]-N-MSCP for 1 hour at 37°C. [^{3}H]-N-MSCP binding to MRs from brain cortex is greater ($p < 0.05$) in animals treated with Cu^{2+} (gray diamond) and PA (black square) as compared with control group (open circle). Data are the mean ± standard error calculated from 6 independent data points.

In all regions examined, Hill's coefficient (n_H) values were greater than 1, which suggests positive homotropic cooperativity in the binding of [^{3}H]-QNB to the MRs; however, no differences were observed among groups. Likewise, there were not significant differences in the dissociation constant (Kd) for all tissues studied, suggesting that both Cu^{2+} and PA did not affect receptor affinity (Table II).

Locomotor activity, rearing behaviour and memory efficiency

After 24 hours from the last doses of Cu^{2+} or PA, we tested locomotor activity, rearing behaviour, and memory. Results indicated that chronic administration of PA induced a significant decrease of locomotor's activity (control rats: 14.7 ± 1.23 events/10 min, PA rats: 7.5 ± 1.12 events/10 min) and rearing behaviour (control rats: 25.5 ± 2.26 events/10 min, PA rats: 10.1 ± 2.12 events/10 min). On the other hand, PA increases significantly memory efficiency (control rats: 17.85 ± 6.7% of success/sec, PA rats: 23.59 ± 5.07% of success/sec). Cu^{2+} decreased significantly the rearing behaviour (16.3 ± 0.91 events/10 min), but no significant difference was observed on locomotor's activity and memory efficiency (Table III).

Cu^{2+} concentrations measured in brain tissue

We only determined Cu^{2+} levels in cortex because of the tissue sample's size required for this type of analysis in our experimental conditions. As expected, Cu^{2+} con-

TABLE II
[^{3}H]-QNB DISSOCIATION CONSTANT AND HILL'S COEFFICIENT IN HOMOGENATES FROM CORTEX AND BRAINSTEM

Treatment	Cortex		Brainstem	
	Kd	n_H	Kd	n_H
Control	107 ± 1.1	1.92 ± 0.1	77 ± 23	1.65 ± 0.05
Cu^{2+}	112 ± 3.1	1.98 ± 0.1	76 ± 8.3	1.67 ± 0.03
PA	115 ± 2.7	2.05 ± 0.1	66 ± 1.4	1.86 ± 0.17

Data are the mean ± standard error (n = 6 for each group). Dissociation constant (Kd) are expressed in picomolar and n_H was calculated based on Hill's equation using Graphpad Inplot software.

TABLE III
BEHAVIOUR PARAMETERS

Type of behavior	Control	Cu^{2+}	PA
Locomotor activity	14.7 ± 1.23	12.0 ± 1.30	7.5 ± 1.12*
Rearing behaviour	25.5 ± 2.26	16.3 ± 0.91*	10.1 ± 2.12*
Memory efficiency	17.85 ± 6.7	15.7 ± 6.55	23.59 ± 5.07*

Locomotor activity and rearing behaviour are expressed as the mean of the number of events in 10 minutes ± standard error (n = 10 for each group). Memory efficiency is expressed as a percentage of success by unit time in seconds. *$p < 0.05$ by ANOVA followed by Bonferroni' post test.

centration was significantly higher ($p<0.05$) in the cortex of rats treated with Cu^{2+} (3.24 ± 0.17 μg/g) as compared with the control groups (2.54 ± 0.54 μg/g); however, PA treated rats, also displayed higher concentrations of copper as compared with control animals (3.01 ± 0.26 μg/g) (Table IV).

TABLE IV
TISSUE LEVELS OF Cu^{2+} IN CORTEX

Treatments	[Cu^{2+}]
Control	2.54 ± 0.54
Cu^{2+}	3.24 ± 0.17 *
PA	3.01 ± 0.26 *

Determined by AAS, expressed in g/g of tissue. Values are the mean ± S.E.M (n = 10 for each group. * Different from control group, values $p < 0.05$ (ANOVA followed by Bonferroni' post test)

DISCUSSION

Copper is a transition metal of broad distribution in mammalian tissues including the central nervous system (26, 27). Brain's areas of high Cu^{2+} content (i.e., hippocampus and striatum) are described also have a high content of muscarinic synapses, which are highly sensitive to alterations in the Cu^{2+} homeostasis (16).

There are few reports on the neurological effects of Cu^{2+} administration in experimental animals. Oral administration of Cu^{2+} affected neither locomotor activity nor learning and/or memory abilities; however, Cu^{2+} in combination with divalent manganesum (Mn^{2+}) impaired learning ability and memory (28). Analysis of bioorganic amines, in the brains of animals receiving these cations, revealed significant increment in the levels of dopamine and noradrenaline levels (28, 29). These findings suggested that Cu^{2+} increased the release of dopamine, noradrenaline, and 5-hydroxytryptamine and inhibited the reuptake of dopamine and 5-hydroxytriptamine in striatum and cortex synapses (30). Similarly, Cu^{2+} deficiency decreased both dopamine and noradrenaline levels (22).

In this report, we showed for the first time, that intraperitoneal administration of high doses of Cu^{2+} results in the induction of up-regulation of the MR number in striatum, cerebral cortex, and hippocampus, but it did not affect the MR population in brainstem. On the other hand, administration of the Cu^{2+}-chelator PA induced MR up-regulation only in the striatum and cerebral cortex. Both, PA and Cu^{2+} treatment increased the Cu^{2+} levels in cerebral cortex, suggesting that the MR up-regulation might be related to the increased copper levels in the selected brain's areas.

It has been reported, that Cu^{2+} inhibits the binding of antagonists to MRs *in vitro* experiments (12, 15, 16), but increases the binding of agonists (12, 16-19). In agreement with reports of agonist-induced

MR down-regulation (31), the administration of Cu^{2+} might induce MR down-regulation by facilitating endogenous agonist binding however, this phenomenon was not observed in our experiments. On the contrary, we found that administration of Cu^{2+} promotes up-regulation of the MR population.

A possible mechanism for the observed Cu^{2+} effect on the MR population could involve its known affinity for cystein moieties and its reducing capacity. Once bound, Cu^{2+} is able to modify the cysteine's sulfhydryl groups' redox state (18). Modifications of these groups could result in marked changes in muscarinic agonist's binding affinity (17, 18).

On the other hand, Cu^{2+}-reduction of sulfhydryl groups may protect the receptors to be desensitized and degraded as is seen when agonists bind the MR inducing receptor phosphorylation and subsequent receptor degradation (31). Supporting this concept, site-direct mutagenesis studies have demonstrated the role of the cysteine residues at the carboxyl-terminus of several transmembrane receptors. These residues are described to be critical for functional desensitization and/or for internalization of alpha adrenergic and glucose-dependent insulinotropic receptors (32-34). For this reason, the up-regulation of MR density on the described Cu^{2+}-responsive brain's areas could be explained by a decreased in the degradation rate of the MRs. This observation is sustained by former observations that rats fed with Cu^{2+}-deficient diets displayed a decrease in the number of receptors at those brain regions (20, 21). These results suggest that Cu^{2+} regulates MRs *in situ* by altering the receptor's redox state and therefore, increasing the half-life of the receptor.

On the other hand, Cu^{2+} could modulate the MR gene expression. Cu^{2+}-mediated activation of yeast's gene expression occurs through Cu^{2+}-regulation of DNA binding regions. Cu^{2+} binding stabilizes a DNA-specific conformation, favoring high affinity interaction between the receptor's specific DNA promoter region and transcriptions elements (35).

Similarly, we observed that PA treatment induced up-regulation of MRs in most of the brain's areas studied, which is correlated with an increased Cu^{2+} tissue level. PA is used in the treatment of Wilson's disease, which is characterized by a generalized hypercuprosis (36). In Wilson's disease patients receiving PA, the half-life to decrease Cu^{2+} concentrations in the cerebro-spinal fluid is about 23.5 months (37). During the first months of treatment, PA seems to worsen the neurological manifestations of the disease, this phenomenon has been explained in relationship to a disturbance of blood-brain barrier (37).

In our protocol, experimental animals received PA for 1.5 months, which might be not enough time to decrease the total brain's copper levels, although sufficient to mimic the PA-induced worsening of neurological conditions. Systemic PA treatment may induce Cu^{2+} redistribution from peripheral tissues to the brain aided by a disruption on the blood-brain barrier integrity allowing for easier access of Cu^{2+} to the central nervous system and inducing a transient hypercuprosis. We do not have a plausible explanation for the effect observed in hippocampus, where Cu^{2+} failed to induce an up-regulation of the MR population; although this observation will be object of further studies, we speculate that hippocampus might have a higher sensitivity to PA treatment that might be promoting a reduction in the hippocampus's Cu^{2+} concentrations earlier.

Cerebral cortex and striatum responded similarly to Cu^{2+} and PA treatment: increase in copper concentrations and up-regulation of MR population; how-

ever, Cu^{2+} and PA treatments did not translated in comparable behavior modifications. While copper treatment affected only rearing behaviour, PA produced a characteristic cholinergic-like behaviour, characterized by hypomotility (decrease in horizontal and vertical displacements) and a better performance in memory trials. In order to analyze this late result, it is necessary to consider that the animals receiving PA, also received zinc chloride and vitamin B6 orally. Zn^{2+} is described to have a variety of neuromodulatory effects *in vitro*, including inhibition of NMDA and GABA A receptors, potentiation of AMPA receptor responses, increased release of GABA, and inhibition of the glutamate transporter EAAT1 (38). Furthermore, reduced dietary Zn^{2+} intake or administration of Zn^{2+} chelators into the brain has shown to induce behavioral changes, including an impaired spatial learning, working memory, and nociception (38, 39). We suggest therefore, that the effect observed in PA-treated animals might be due an induced imbalance between Cu^{2+} and Zn^{2+} in favor of a Zn^{2+} -mediated effect.

Finally, our data showed that Cu^{2+} upregulates muscarinic cholinergic receptors in selected brain areas. Although Cu^{2+} by itself does not seem to affect animal's behavior, the impact of Cu^{2+}-induced MR upregulation on the behavior responses might be related to the levels of Zn^{2+} in the central nervous system.

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