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Pharmacological Doses of Zn^{2+} Induce a Muscarinic Cholinergic Supersensitivity

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Key Words

Zn^{2+} · Muscarinic receptor · Memory · Motility · Rearing · [^{3}H]-QNB · Binding · Cholinacetyltransferase · Cholinergic · Brain

Abstract

The goal of this study was to evaluate the effect of chronic Zn^{2+} administration (1 mg/kg/day for 1 month) in Sprague-Dawley rats (n = 11) on motility and rearing behaviors (number of events/10 min measured in motility cage), on memory (percentage of failures using a foot-shock double T maze), on the number of muscarinic receptors (using [^{3}H]-QNB as a marker) and on the cholinacetyltransferase (Chat) activity (determined by Fonnun's method) in various brain areas (striatum, hippocampus and frontal cortex), as compared with saline-treated rats (n = 10). Our results showed that Zn^{2+} induced a decrease in rearing (control: 24.6 ± 3; Zn^{2+}: 15.91 ± 2.19) and in locomotor activity (control: 37 ± 3.79; Zn^{2+}: 25 ± 4.37), a decrease in failures during memory trials (control: 26.12 ± 5.6; Zn^{2+}: 5.33 ± 2.71) and an increase in muscarinic receptor density (fmol/mg) in the striatum (control: 539 ± 6.18; Zn^{2+}: 720 ± 14.69), hippocampus (control: 396 ± 7.41; Zn^{2+}: 458 ± 5.05) and frontal cortex (control: 506 ± 10.28; Zn^{2+}: 716 ± 16.54). Chat activity (pmol/mg/min) was decreased only in the striatum (control: 4,240 ± 158; Zn^{2+}: 2,311 ± 69). We conclude that Zn^{2+} induces a cholinergic functional supersensitivity which is related to receptor upregulation.

Introduction

Muscarinic cholinergic receptors belong to a superfamily of receptors with seven transmembrane helix domains which are coupled to G proteins [3, 5, 13]. Five muscarinic receptor subtypes have been described based on molecular cloning studies: m1, m2, m3, m4 and m5. Four of these cloned subtypes have been pharmacologically and functionally defined in primary tissues, corresponding to the functional receptors identified as M1, M2, M3 and M4 [5].

Cholinergic neurotransmission has been shown to be involved in a variety of centrally mediated brain functions, such as learning, memory and cognition, besides its classical role in peripheral neurotransmission [2, 11, 32]. Loss of cholinergic integrity, including decreased brain levels of cholinacetyltransferase (Chat), loss of cortical

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cholinergic projections on the forebrain and a reduced number of muscarinic receptors have been demonstrated in patients with Alzheimer's disease, which is the most prevalent memory disorder [17]. Furthermore, muscarinic receptor antagonists can disrupt acquisition and performance of learned behaviors via the blockage of the interaction between acetylcholine and its muscarinic receptors [4].

Several endogenous factors have been reported to be able to modify functions of muscarinic receptors, among them, putative endogenous regulatory ligands [1, 27], guanine nucleotides [19] and ions such as Zn^{2+} and Cu^{2+} [20].

Zinc (Zn^{2+}) is an essential dietary trace element which plays an important role in neurotransmission by modulating the activity of glutamate and GABA receptors [31]. It has been suggested that Zn^{2+} plays a pathological role in neurological disorders such as epilepsy, ischemia and Alzheimer's disease [7].

Zn^{2+}-containing neurons/nerve terminals have been identified in several regions of the brain, including the hippocampus, cortex and cerebellum [10]. The hippocampus contains the highest concentration of Zn^{2+} [34]. The hippocampus is a region that participates in spatial learning and memory [21], and it is expected that Zn^{2+} may be involved in regulating hippocampals function. Consistent with this line of thought, it has been reported that administration of Zn^{2+} or Zn^{2+} chelators to animals affects spatial learning, working memory and nociception; addition of Zn^{2+} to hippocampal slices results in impaired long-term potentiation (LTP) [34, 35]. In humans, a significant correlation between dietary Zn^{2+} and students' attention-seeking behavior and activity level in the classroom has been shown [14].

The effect of Zn^{2+} on muscarinic receptor function has been explored only in in vitro studies. Zn^{2+} is able to inhibit the binding of L-quinuclidinyl[phenyl-4-^{3}H]-benzilate ([^{3}H]-QNB) to human [8] and rat brain membrane preparations [22], and it was required to disclose the inhibition of binding of [^{3}H]-QNB exerted by an endogenous inhibitor [9]. In like manner, it has been shown that Zn^{2+} blocks muscarinic receptor activation-induced Ca^{2+} release from intracellular stores [24–29]. No studies have been performed relating the modulating effect of Zn^{2+} on muscarinic receptors to behavioral changes in learning, memory and/or motility.

In spite of the knowledge relating to the in vitro effect of Zn^{2+} on muscarinic receptor function, the behavioral consequences of Zn^{2+}-induced muscarinic modulation have not yet been studied. For this reason, we decided to assess the effects of chronic administration of Zn^{2+} on learning, memory and locomotor activity of young Sprague-Dawley rats, followed by determination of muscarinic receptor-binding properties and Chat activity, in order to correlate molecular with behavioral findings. Our results showed that Zn^{2+} treatment induced upregulation of muscarinic receptors. These molecular findings were related to cholinergic behavior supersensitivity expressed as hypomotility and increased memory performance.

Materials and Methods

Animals and Treatments

Two groups of 21-day-old male Sprague-Dawley rats were injected intraperitoneally daily for 30 days with either 1 mg/kg $ZnCl_2$ (experimental group; n = 11) or an equal volume of 0.85% NaCl (control group; n = 10). After treatments, all rats were assayed for memory and locomotor activity. After that, they were sacrificed by decapitation, and the brains were rapidly removed. Homogenates were prepared (1/20, w/v) in 25 m*M* Hepes buffer, pH 7.3, 1 m*M* EDTA and 1 m*M* PMSF, aliquoted in small volumes and stored at –70°C until used.

Binding Assays

250 µg of brain homogenate protein was incubated for 2 h at 37°C with 500 p*M* [^{3}H]-QNB, in a final volume of 2.5 ml, completed with 25 m*M* Hepes buffer (pH 7.3) containing 0.1 m*M* PMSF in the absence (total binding) or presence (nonspecific binding) of 2 µ*M* atropine at 37°C for 60 min. Binding reactions were terminated by vacuum filtration through GF/B glass-fiber filters (Whatman Inc., Clifton, N.J., USA). Filters were washed three times with 5 ml of ice-cold phosphate buffer, dried (12 h at 60°C) and placed into scintillation vials with 5 ml of scintillation cocktail (PPO, POPOP, Triton X-100 and toluene). Incorporated radioactivity was measured by a liquid scintillation counter (50% counting efficiency; Wallac 1410, Pharmacia Inc., Finland). Nonspecific binding values were subtracted from total binding values to give specific binding. Protein concentration was determined by bicinchoninic acid colorimetric assay.

Chat Activity

Chat activity was measured according to the radiometric method of Fonnun. Briefly, tissue homogenates were prepared (1/10, w/v) in 10 m*M* EDTA (pH 7.4), treated with Triton X-100 (final concentration 0.5%) for 15 min at room temperature and centrifuged at 20,000 *g* for 15 min. 10 µl of the supernatant was incubated with 22 µl of a cocktail substrate solution containing 0.73 m*M* acetyl coenzyme A (AcCoA) and 0.46 m*M* eserine sulfate in buffer substrate (1.2 *M* NaCl, 200 m*M* NaH_2PO_4, 32 m*M* choline chloride, 35 m*M* EDTA, pH 7.4). Enzymatic reaction was initiated by adding 40 nCi of [^{3}H]-AcCoA and incubating for 15 min at 37°C, and stopped by addition of 5 ml of ice-cold 10 m*M* EDTA buffer (pH 7.4). The [^{3}H]-acetylcholine formed during the reaction was extracted by 2 ml of kalignost solution (5 mg/ml sodium tetraphenylborate dissolved in acetonitrile) and added to 10 ml of scintillation cocktail. Radioactivity was measured by a liquid scintillation counter.

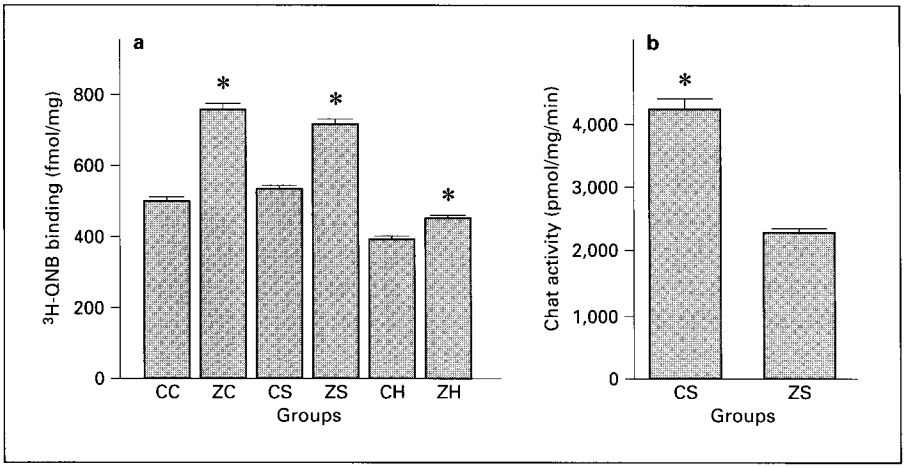

Fig. 1. Number of muscarinic receptors and Chat activity. **a** 250 µg of homogenate protein from different brain regions, i.e. frontal cortex (C), striatum (S) and hippocampus (H), were obtained from saline (control; C)- or Zn^{2+} (Z)-treated rats and incubated with 500 p*M* [^{3}H]-QNB for 2 h at 37 °C in 25 m*M* Hepes buffer. Brain homogenates from Zn^{2+}-treated rats displayed a higher muscarinic receptor density in all brain areas as compared with the control group (* $p < 0.05$). **b** Chat activity displayed a significant decrease only in the striatum. CS = Striatum from control rats; ZS = striatum from Zn^{2+}-treated rats. * $p < 0.05$.

Behavioral Testing

After 2 days of habituation trials, consisting of permitting the rats to explore the maze freely for 30 min (one trial per day), learning and memory were measured using a foot-shock double T maze with 20 s of latency time. The performance of each rat on the first day was considered as learning, and successive performances on the following days were considered as memory. For each rat, the percentage of failures which occurred in solving the maze on each daily test was scored; each test consisted of 10 trials. A failure was considered to have occurred whenever the rat did not solve the maze or entered a wrong arm. Locomotor activity was measured using a motility cage divided into four chambers; simultaneously, rearing behavior was scored. Motility activity was recorded for a period of 10 min, preceded by a latency period of 5 min.

Data Analysis

Data are expressed as the mean ± standard error. Statistical analysis of the differences between groups was performed using Student's t test, accepting $p < 0.05$ as significant.

Materials

[^{3}H]-QNB, with a specific activity of 48 Ci/mmol, and [^{3}H]-AcCoA, with a specific activity of 4.8 Ci/mmol, were purchased from Amersham Pharmacia Biotech (UK) and NEN Life Science Products (USA), respectively. Atropine sulfate and $ZnCl_2$ were obtained from Sigma Chemical Company (USA).

Results

Muscarinic Receptors

Homogenates prepared from Zn^{2+}-treated rats displayed a higher muscarinic receptor density in the cortex (716 ± 16.54 fmol/mg), striatum (720 ± 14.69 fmol/mg) and hippocampus (458 ± 5.05 fmol/mg) than control groups (506 ± 10.28, 539 ± 6.18 and 396 ± 7.41 fmol/mg in cortex, striatum and hippocampus, respectively) (fig. 1a, table 1).

Table 1. Muscarinic receptor densities in different brain areas

Structure	Control	Zn^{2+}
Cortex	506 ± 10.28	716 ± 16.54*
Striatum	539 ± 6.18	720 ± 14.69*
Hippocampus	396 ± 7.41	458 ± 5.05*

Values are femtomols of [^{3}H]-QNB bound per milligram of protein. * $p < 0.05$ by Student's t test.

Locomotor Activity, Rearing Behavior and Memory

Twenty-four hours after the last doses of Zn^{2+}, locomotor activity, rearing behavior and memory were tested. Results indicated that chronic administration of Zn^{2+} induced a significant decrease in rearing (control rats: 24.6 ± 3 events/10 min; Zn^{2+} rats: 15.91 ± 2.19 events/10 min) (fig. 2b) and locomotor activity (control rats: 37 ± 3.79 events/10 min; Zn^{2+} rats: 25 ± 4.37 events/10 min) (fig. 2a). On the other hand, Zn^{2+} induced a decrease in the number of failures in three consecutive memory trials (control rats: 26.12 ± 5.6% failures; Zn^{2+} rats: 5.33 ± 2.71% failures) (fig. 2c).

Chat Activity

Chat activity was significantly decreased in solubilized homogenates from striatum of rats treated with Zn^{2+}, which exhibited an activity of 2,311 ± 69 pmol/mg/min,

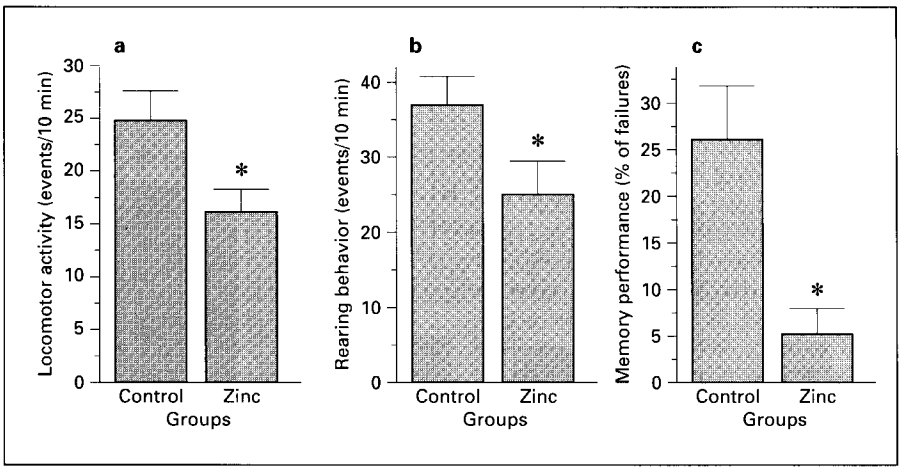

Fig. 2. Behavioral effects of Zn^{2+}. **a**, **b** Twenty-four hours after the treatment, each rat was put individually in a motility cage (60 × 60 × 30 cm) divided into four chambers. Horizontal (locomotor activity; **a**) and vertical (rearing; **b**) displacements were measured during a 10-min period after 5 min of latency time. Zn^{2+}-treated rats displayed a significant decrease in both motor behaviors with respect to the control group (* $p < 0.05$). **c** After 2 days of habituation trials, learning and memory were measured using a foot-shock double T maze with 20 s of latency time. Memory for each rat was scored as the percentage of failures which occurred in solving the maze for each daily test. Rats treated with Zn^{2+} showed a decrease in the number of failures as compared with rats in the control group (* $p < 0.05$).

Table 2. Chat activity in different brain areas

Structure	Control	Zn^{2+}
Frontal cortex	2,089 ± 109	1,767 ± 172
Striatum	4,240 ± 158	2,311 ± 69*
Hippocampus	1,743 ± 99	1,586 ± 41

Values are picomols of [^{3}H]-acetylcholine produced per milligram of protein per minute. * $p < 0.05$ by Student's t test.

while the control group displayed an activity of 4,240 ± 158 pmol/mg/min (fig. 1b, table 2). No significant differences were observed in the enzyme activity of solubilized homogenates obtained from frontal cortex and hippocampus of Zn^{2+}-treated rats as compared with solubilized homogenates obtained from the control group (table 2).

Discussion

In the present paper, we have presented data supporting the idea that Zn^{2+} is able to induce brain muscarinic receptor supersensitivity and in this manner is able to increase memory performance in fear-conditioning trials.

Zn^{2+} is believed to be an endogenous modulator that regulates learning and memory functions [16]. Zn^{2+} is particularly able to regulate glutamate excitatory synaptic transmission [29]. In the CA3 region of the hippocampus, there is an abundance of zinc, which is located in presynaptic mossy fiber nerve terminals [10]. Stimulation of these fibers can cause the release of Zn^{2+}, which interacts with excitatory amino acid receptors and may therefore modulate LTP [34]. LTP is promulgated as an example of a learning and memory mechanism at the synaptic level. Incubation of hippocampal slices with Zn^{2+} resulted in an inhibition of LTP induced by mossy fiber stimulation in CA3 neurons [35].

In the present study, we observed that chronic administration of Zn^{2+} (1 mg/kg) significantly decreased the percentage of failures in foot-shock double T maze memory trials. These results are in agreement with studies that have shown that a deficiency of Zn^{2+} can inhibit memory [30]. Thus, memory deficit induced by Zn^{2+} deficiency has been shown to be reverted by repletion with dietary Zn^{2+} [16]. Likewise, reversible binding of Zn^{2+} by infusion of chelating drugs into the hippocampus produces a time-locked and selective disruption of hippocampal-dependent spatial-working memory [12]. In addition, severe Zn^{2+} deficiency during lactation results in offspring who suffer a spatial learning deficit and a working memory impairment [15]. On the other hand, it has been

reported that administration of high doses of Zn^{2+} to rats results in a spatial reference memory deficit [30], an effect that could be related to a neurotoxic effect exerted by zinc [33].

Cholinergic neurotransmission has been implicated in the processes of learning and memory, administration of muscarinic receptor antagonists or deafferentation of cholinergic input to hippocampus; both result in memory and attentional deficits [26]. Muscarinic transmission is also implicated in disease states related to aging, and degeneration of cholinergic basal forebrain neurons which project to the cortex and hippocampus in Alzheimer's disease has been suggested to contribute to the dementia syndrome [17]. The mechanism by which muscarinic receptors are involved in learning and memory is still not known.

Zn^{2+} is able to inhibit the binding of [^{3}H]-QNB to the muscarinic receptors [8, 22]; similarly, the endogenous inhibitor of a muscarinic receptor required Zn^{2+} to display its activity [9]. These data suggest that Zn^{2+} could behave as a modulator of muscarinic function. In the present study, we observed that chronic administration of Zn^{2+} induced cholinergic receptor upregulation in the striatum, frontal cortex and hippocampus, and we also observed that Chat activity was depressed in the striatum. Loss of Chat enzyme activity in the striatum could have been related to a degeneration of presynaptic cholinergic projections to this region caused by Zn^{2+}. It is known that elevated brain levels of Zn^{2+} are neurotoxic, especially to cerebellar granule cells and cortical neurons [33]. Degeneration of presynaptic cholinergic neurons could explain the muscarinic receptor upregulation observed in the striatum; however, this proposition cannot be used to explain the phenomena that were observed in the frontal cortex and hippocampus, because in these regions, Chat enzyme activity was unchanged in the Zn^{2+}-treated rats. In this case, a muscarinic cholinergic upregulation could be the consequence of a decrease in acetylcholine release due to Zn^{2+}. It has been reported that Zn^{2+} is able to partially block catecholamine release in PC12 cells due to a capacitive Ca^{2+} entry block [28]; similarly, the calcium influx pathway has been shown to be blocked by Zn^{2+} in rat neocortical neurons and glial cells [24].

A decrease in acetylcholine release must be reflected as a cholinergic hypofunction, but in our experiment, we observed a clear cholinergic supersensitivity, characterized by an increase in memory performance and a decrease in rearing and motility behavior (fig. 2), indicating that cholinergic synaptic transmission was strengthened. Therefore, muscarinic receptor upregulation should be analyzed in terms of an increase in gene expression or a decrease in receptor desensitization and internalization.

A Zn^{2+} finger motif provides a DNA-binding domain to certain types of transcription factors. This motif has a conserved amino acid sequence that binds zinc ion. Zn^{2+} is held in a tetrahedral structure formed by cysteine and histidine residues. The transcription factors regulate gene expression binding to this motif [18]. It is interesting to note that the promoter for the M2 muscarinic receptor gene contains a site for the GATA family of transcription factors, which is required for a high level of gene expression; GATA family members contain DNA-binding domains with two zinc fingers [25]. Dietary Zn^{2+} deficiencies cause a decrease in gene expression for different protein systems, measured as protein and/or RNA levels [6, 23].

Finally, a decrease in the degradation rate of muscarinic receptors could explain the observed upregulation. Zn^{2+} could regulate muscarinic receptors 'in situ' by an allosteric mechanism or by regulating the receptor redox state, and therefore increasing the half-life of the receptor.

In conclusion, Zn^{2+} induced a cholinergic functional supersensitivity which could be related to muscarinic receptor upregulation in the rat brain.

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