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Supplementation with Zinc in Rats Enhances Memory and Reverses an Age-Dependent Increase in Plasma Copper

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Abstract

Zinc and copper are essential trace elements. Dyshomeostasis in these two metals has been observed in Alzheimer's disease, which causes profound cognitive impairment. Insulin therapy has been shown to enhance cognitive performance; however, recent data suggest that this effect may be at least in part due to the inclusion of zinc in the insulin formulation used. Zinc plays a key role in regulation of neuronal glutamate signaling, suggesting a possible link between zinc and memory processes. Consistent with this, zinc deficiency causes cognitive impairments in children. The effect of zinc supplementation on short- and long-term recognition memory, and on spatial working memory, was explored in young and adult male Sprague Dawley rats. After behavioral testing, hippocampal and plasma zinc and copper were measured. Age increased hippocampal zinc and copper, as well as plasma copper, and decreased plasma zinc. An interaction between age and treatment affecting plasma copper was also found, with zinc supplementation reversing elevated plasma copper concentration in adult rats. Zinc supplementation enhanced cognitive performance across tasks. These data support zinc as a plausible therapeutic intervention to ameliorate cognitive impairment in disorders characterized by alterations in zinc and copper, such as Alzheimer's disease.

Keywords

Zinc; copper; memory; hippocampus

1. Introduction

Zinc regulates many cellular processes, acting as a co-factor for more than 300 enzymes [1–8]. With only 2–4g of zinc within the adult human body, concentration of zinc is tightly regulated [9]. Within the brain, zinc is concentrated in the limbic system [2, 6, 10], predominantly in the hippocampus and amygdala [2, 11–13]; the hippocampus is the only brain area in which zinc increases markedly during development [14]. Zinc deficiency impairs cognitive and motor function in children [15, 16] and damages the blood brain

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barrier [17], but less is known about the relationship between zinc and adult memory. Disturbances in zinc homeostasis have been associated with aging [18], Type II Diabetes (T2D)[9, 19–22], and Alzheimer's disease (AD)[23–35], and this zinc dysregulation may contribute to the cognitive dysfunction seen in these disease states [36].

Zinc is co-secreted with glutamate. Zinc-containing glutamatergic neurons are dense in the mossy fibre layer of the hippocampus [1, 5, 37, 38]: a zinc-deficient diet causes reduced neurogenesis and increased neuronal apoptosis within the hippocampus [39–42]. Vesicular zinc has recently been shown to be critical for hippocampal long-term potentiation (LTP) with both presynaptic and postsynaptic actions [43]. After secretion, zinc modulates postsynaptic excitability at NMDA [44–47], dopamine [48], and GABA receptors [49, 50]; zinc also modulates the Erk1/2 mitogen-activated-protein kinase (MAPK) pathway[51, 52], brain-derived neurotrophic factor (BDNF)[10, 53, 54] and glucose metabolism [55, 56]. An additional zinc-dependent enzyme recently identified as a potential modulator of hippocampal learning and memory is insulin-regulated aminopeptidase (IRAP) [57–60].

Since it was shown that zinc is a component of insulin crystals [61], a relationship between zinc and insulin signaling, a pathway known to impact learning and memory [62–64], has been proposed. More specifically, zinc causes tyrosine phosphorylation of the β subunit of the insulin receptor, increases phosphorylation of Akt serine residues and therefore activation of Akt, and induces an increase in glucose transport into cells via GLUT4 translocation [9, 65–67].

Despite these several known effects of hippocampal zinc, zinc's role in adult cognitive impairment has not been extensively explored. Reduced zinc signaling produced by knock-out of the ZnT3 zinc transporter (responsible for packaging of vesicular zinc) produces cognitive impairment in 6 month-old mice, accompanied by marked glutamatergic dysfunction and a decrease in total dendritic spines per neuron [26] but this cognitive impairment was, interestingly, absent in young (6–10 week old) ZnT3 KO mice, suggesting that zinc regulation may be especially important in adult or aged brains.

Recent research [68, 69] suggests a possible link between zinc and a second essential micronutrient, copper, in the development and/or progression of Alzheimer's disease [30, 33, 70–76]. Copper can be toxic when in excess by contributing to oxidative stress [77]. Given this possible link, we measured both brain and plasma copper following zinc administration. Our overall hypothesis was that zinc supplementation might enhance hippocampally-mediated cognitive function, possibly via modulation of copper levels; and that this effect would be seen more clearly in adult rats than in young rats.

2. Material and Methods

2.1. Participants

Male Sprague Dawley rats (N=40; Charles River, Wilmington, MA), either 8-weeks-old (young group) or 24 weeks old (adult group), were pair-housed on a 12:12 h light:dark schedule with food and water available *ad libitum*. All procedures were approved by the University at Albany Institutional Animal Care and Use Committee. All rats were allowed to

acclimate for at least one week prior to treatment. Animals were handled routinely from the time of their arrival by the experimenters to minimize any effects of handling stress on experimental measurements.

2.2 Zinc supplementation

Animals were randomly assigned to either regular drinking water or zinc-supplemented drinking water (75 mg/L elemental zinc) for two-weeks prior to behavioral testing. Animals remained on their assigned treatments during the behavioral testing schedule, totaling three weeks of treatment at the time of brain and blood collection.

2.3 Behavioral testing

Open Field—Animals first completed an Open Field (OF) task. OF testing involved placing the animal into an open arena for 5 minutes. Time spent in the center zone and outer zone of the arena is recorded, with increased time in the center zone indicating lower anxiety. This task also served as a habituation trial for subsequent novel object recognition testing. The OF apparatus was cleaned with 70% ethanol in-between trials, ensuring no carry-over effects of scent influenced subsequent testing.

Novel Object Recognition—Thirty minutes after performing OF, animals performed Novel Object Recognition (NOR) to assess short- and long-term recognition memory. NOR testing involved placing the animals back into the open arena used in OF testing for three more 5-minute trials. During the first trial, two identical objects were placed in the center zone and total time exploring both objects was recorded. Thirty minutes later, one of the identical objects was replaced with a novel object and the animals were returned to the behavioral apparatus. Time spent exploring the novel object as a percentage of total exploration time was calculated as 30-minute novel object recognition. Twenty-four hours later, the animals were returned to the behavioral apparatus a second time, and a different novel object again replaced one of the identical objects seen during acquisition; a 24-hour novel object recognition score was calculated in the same way. Higher scores are interpreted as indicating increased recognition memory. The objects used during NOR testing included a pair of water-filled 500 mL conical bottles, an opaque white teacup, and laboratory goggles. The OF apparatus and objects were cleaned with 70% ethanol in-between trials, ensuring no carry-over effects of scent influenced subsequent testing.

Spontaneous Alternation—Forty-eight hours after completing NOR, animals were tested on the 4-arm Spontaneous Alternation (SA) spatial working memory task [62, 78, 79]. This task is known to be sensitive to alterations in hippocampal metabolism and insulin signaling [63, 80–84]. Animals were placed in a 4-arm plus-shaped maze in which the animals utilized spatial cues to guide behavior. Alternation was calculated as the percentage successful alternations (entering each arm at least once in 5 attempts) of total possible alternations (number of arm entries minus four). Higher alternation scores indicate increased spatial working memory. The SA apparatus was cleaned with 70% ethanol in-between trials, ensuring no carry-over effects of scent influenced subsequent testing.

2.4 Atomic Absorption Analysis

Trunk blood and hippocampal tissue were collected at the time of sacrifice. Blood was immediately spun down and separated for plasma while hippocampi were removed and immediately homogenized and fixed in protease and phosphatase inhibitor buffers to prepare the tissue for atomic absorption (AA; Aurora Biomed Trace AI 1200) analyses. Plasma and homogenate were diluted with HPLC grade water to reduce background absorption. Zinc and copper concentrations were quantified using associated software relative to respective standard curves.

2.5 Statistical Analyses

A 2×2 (age $\times$ treatment) ANOVA assessed the effects of age (8-weeks vs. 24-weeks) and treatment (control vs. zinc-supplemented), and the interaction of age and treatment, on open field, novel object recognition at 30-minute and 24-hour latency, and spontaneous alternation. A separate 2×2 (age $\times$ treatment) ANOVA assessed effects on plasma zinc, hippocampal zinc, plasma copper, and hippocampal copper. Significant main effects and interactions were further examined using post-hoc pairwise comparisons.

Statistical outliers were defined as falling more than two standard deviations from the mean for behavioral and biological analyses. If an animal was determined an outlier during the acquisition phase of NOR, subsequent trials (30-minute and 24-hour latency) were also removed from analyses ($N=5$). If an animal was removed at any point during behavioral testing, it was also removed for biological analysis ($N=7$; $N=1$ removed from OF, $N=5$ from NOR, $N=1$ from SA). Samples that fell outside of the standard curve range during biological analysis and/or were more than two standard deviations from the mean were also removed ($N=6$). One 24-week-old animal was removed from the study prior to treatment due to health problems, resulting in a total of $N=39$ for behavioral and biological analyses prior to removal of statistical outliers and samples that fell outside of the standard curve. Group sizes for each analysis are as follows, Young Control: Open Field $n=10$, NOR $n=8$, SA $n=10$, Plasma Zinc $n=8$, Plasma Copper $n=8$, Brain Zinc $n=8$, Brain Copper $n=8$; Young Zinc: Open Field $n=9$, NOR $n=10$, SA $n=10$, Plasma Zinc $n=7$, Plasma Copper $n=7$, Brain Zinc $n=7$, Brain Copper $n=7$; Adult Control: Open Field $n=10$, NOR $n=9$, SA $n=10$, Plasma Zinc $n=6$, Plasma Copper $n=6$, Brain Zinc $n=7$, Brain Copper $n=7$; Adult Zinc: Open Field $n=9$, NOR $n=7$, SA $n=8$, Plasma Zinc $n=6$, Plasma Copper $n=7$, Brain Zinc $n=7$, Brain Copper $n=7$. Alpha was set at 0.05; data are shown as group means $\pm$ SEM.

3. Results

3.1 Behavioral Results

Open Field—No effect of age or treatment on performance in the open field task was seen (all $p > .05$). Group mean times ($\pm$ SEM) spent in the center zone were: Young Control 7.51 $\pm$ 2.03 seconds, Young Zinc 5.00 $\pm$ 1.82 seconds, Adult Control 7.29 $\pm$ 1.11 seconds, Adult Zinc 10.17 $\pm$ 2.15 seconds (data not shown).

Novel Object Recognition—There was no effect of age or treatment, nor their interaction, on time spent exploring objects during acquisition (all $p > .05$; Figure 1A).

Young Control: $M = 55.13$ seconds, $S.E.M. = 7.13$ seconds, Young Zinc: $M = 49.76$ seconds, $S.E.M. = 6.00$ seconds, Adult Control: $M = 41.64$ seconds, $S.E.M. = 7.36$ seconds, Adult Zinc: $M = 46.56$ seconds, $S.E.M. = 7.91$ seconds.

A main effect of treatment was found at both 30-min ($F(1,29) = 68.258$, $p < .05$), and 24-hour latencies ($F(1,29) = 11.275$, $p < .05$) (Figure 1B) on percent novel object recognition. Pairwise post-hoc comparisons confirmed a significant increase in percent novel object recognition after zinc treatment at both 30-minute and 24-hour latencies in the young group, and at the 30-minute latency in the aged group ($p = 0.066$ for the 24-hour latency). There was no significant interaction between age and treatment.

Spontaneous Alternation—No main effect of age or treatment or interaction of age and treatment on percent spontaneous alternation was seen (Figure 1C), $F(1,29) = .093$, $p > .05$ and $F(1,29) = 3.128$, $p > .05$; however, although there was no significant effect of treatment ($F(1,29) = 3.128$, $p = .087$), a pairwise comparison revealed a significant effect of zinc in the adult cohort ($p = .036$) with zinc treatment improving percent spontaneous alternation.

3.2 Atomic Absorption Results

Zinc Concentration—There was a main effect of age on plasma and hippocampal zinc concentration ($F(1,23) = 4.830$, $p < .05$ and $F(1,23) = 7.636$, $p < .05$, respectively; Figure 2A). 24-week-old animals had decreased plasma zinc and increased hippocampal zinc, relative to 8-weeks-old, indicating an age-dependent effect on zinc status. There was no significant interaction between age and treatment. Surprisingly, there was no significant effect of zinc treatment on either plasma or hippocampal zinc levels.

Copper Concentration—There was a main effect of age on plasma and hippocampal copper concentration ($F(1, 23) = 19.037$, $p < .05$ and $F(1,23) = 20.827$, $p < .05$, respectively; Figure 2B). 24-week-old animals had higher plasma and hippocampal copper levels. Interestingly, results also indicated a significant interaction between the two factors ($F(1,23) = 7.817$, $p < .05$), with zinc treatment reducing plasma, but not hippocampal, copper in the 24-week-old animals.

4. Discussion/Conclusion

We show that zinc supplementation enhanced both (i) short- and long-term recognition memory in young rats and short-term recognition memory in adult rats, and (ii) spatial working memory in adult rats. The role of zinc in mnemonic processing has been previously explored [85–88], but this is the first report we are aware of showing an effect of zinc supplementation to enhance memory above baseline.

We also saw increased plasma copper and hippocampal zinc and copper in our adult rats compared to the young animals. Surprisingly, our zinc supplementation did not affect hippocampal or plasma zinc concentrations. One possibility is that reversal of elevated plasma copper by zinc supplementation may contribute to cognitive enhancement, at least in the adult cohort, as excess copper is known to catalyze the formation of highly reactive hydroxyl radicals, initiate oxidative damage, and interfere with important cellular events

[89]. However, zinc also enhanced NOR in the young animals without affecting plasma copper, indicating that there must be additional or secondary effect(s) of zinc treatment beyond that of copper.

Interestingly, while zinc concentration in the brain increases with growth after birth and is maintained constant in the adult brain [90], turnover of zinc in the brain is very slow [91–93], which may help to explain why our two-week supplementation did not significantly alter brain zinc concentration. Consistent with our findings, it was recently shown that a 6-month oral zinc supplementation also had no effect on brain zinc, but did significantly reduce brain copper and amyloid burden in Tg2576 mice [69].

Future studies should include additional measurements of animal weight and water consumption before and during zinc supplementation to understand how possible changes in appetite and/or aversion toward zinc-treated water might produce secondary nutritional effects that could impact memory. These measurements would also aid in determining relative dosing of zinc over the course of a study. Additionally, administration of isotopically enriched zinc would elucidate the transport of zinc supplementation into the brain and determine whether age-related differences in transport exist.

In a prior study of zinc supplementation in patients with Alzheimer's disease, treatment both improved cognitive performance and reduced serum copper [30], similar to our data. AD patients show increased serum and plasma copper relative to aged-matched controls [94, 95], and are zinc-deficient [68]. Patients with AD show high levels of both copper and zinc in cerebrospinal fluid and in amyloid plaques analyzed post-mortem [96, 97]. Taken together with the prior literature, our data in healthy animals suggest the possibility that zinc may be considered as a cognitive enhancer and possible therapeutic for the treatment of cognitive impairment seen in neurodegenerative disorders such as Alzheimer's disease.

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Highlights

- Dyshomeostasis in trace metals such as zinc and copper impact cellular and cognitive processing.
- Zinc supplementation enhances short- and long-term recognition and spatial working memory.
- Hippocampal zinc and copper and plasma copper significantly increase with age.
- Zinc supplementation reverses age-related elevations in plasma copper.
- Zinc supplementation can be considered a plausible cognitive enhancing agent and/or therapeutic for cognitive dysfunction.

Figure 1. Cognitive performance

(A) Time spent exploring objects during novel object recognition acquisition. No group differences were seen, nor were there overall effects of age or treatment. (B) Percent time spent with novel object during testing after a 30min or 24h delay. (C) Percent spontaneous alternation (SA) performance on the 4-arm SA task. Asterisks indicate significant pair-wise differences ($p < .05$) between control and zinc-treated conditions for that group. Data shown as mean $\pm$ SEM.

Figure 2. Zinc and copper measurements

(A) Across treatment conditions, age decreased plasma zinc and increased hippocampal zinc. No effect of zinc treatment was seen on either measure. (B) Age increased both plasma and hippocampal copper. In addition, pair-wise comparison showed that zinc treatment attenuated the age-related increase in plasma copper. Asterisks indicate significant ($p < .05$) effects of age across treatment condition; # indicates significant effect of zinc treatment on plasma copper in the adult animals. Data shown as mean $\pm$ SEM.