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Zinc supplementation in rats impairs hippocampal-dependent memory consolidation and dampens post-traumatic recollection of stressful event

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Abstract
Zinc is a trace element important for synaptic plasticity, learning and memory. Zinc deficiency, both during pregnancy and after birth, impairs cognitive performance and, in addition to memory deficits, also results in alterations of attention, activity, neuropsychological behavior and motor development. The effects of zinc supplementation on cognition, particularly in the adult, are less clear. We demonstrate here in adult rats, that 4 week-long zinc supplementation given by drinking water, and approximately doubling normal daily intake, strongly impairs consolidation of hippocampal-dependent memory, tested through contextual fear conditioning and inhibitory avoidance. Furthermore, the same treatment started after memory consolidation of training for the same behavioral tests, substantially dampens the recall of the stressful event occurred 4 weeks before. A molecular correlate of the amnesic effect of zinc supplementation is represented by a dysregulated function of GSK-3ß in the hippocampus, a kinase that participates in memory processes. The possible relevance of these data for humans, in particular regarding post-traumatic stress disorders, is discussed in view of future investigation.

1. Introduction

Zinc is an essential trace element, important for cell growth, development, proliferation and differentiation, as well as nucleic acid metabolism (Maret and Sandstead, 2006). To carry on its biochemical activity, zinc interacts with hundreds of proteins (Vallee and Falchuk, 1993). A

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special role for zinc in brain function came from the fortuitous observation that intravitreal injection of a zinc chelator heavily labeled hippocampal mossy fibers (Maske, 1955). The introduction of a specific histochemical method (Timm, 1958) confirmed this preferential localization and electron microscopy demonstrated the presence of zinc in synaptic vesicles (Haug, 1967). The reactive metal is free zinc present not only in mossy fibers, but also in presynaptic terminals of cortical, limbic and olfactory brain regions (Friedman and Price, 1984; Frederickson et al., 1987) and represents only a minor fraction of total brain zinc (Vallee and Falchuk, 1993). Zinc is transported inside vesicles by the specific carrier ZnT3 (Palmiter et al., 1996) and in most cases is associated with glutamatergic synapses, although it can also be found in GABAergic synapses (Beaulieu et al., 1992; Sindreu et al., 2003). The presence of high zinc concentrations at synapses, known to be critical for learning and memory, suggested its involvement in synaptic plasticity (Weiss et al., 1989; Sindreu and Storm, 2011). This role has been widely studied over years and many functional interactions of zinc at both pre- and post-synaptic levels have been described. These include membrane and vesicle transporters, ion channels, most notably voltage-gated Ca^{+} $^{+}$ channels, and several neurotransmitter receptors, including NMDA and AMPA glutamate receptors (Sensi et al., 2009; Sindreu et al., 2011). The entrance of zinc through voltage-gated and neurotransmitter-gated channels is followed by its buffering by many proteins, such as metallotioneins, and by its transport into vesicles (Jia et al., 2002; Sheline et al., 2002).

The putative role of synaptic zinc in neural plasticity, and therefore in learning and memory, is supported by several observations demonstrating memory impairment when zinc homeostasis is altered at the synaptic level. Such memory impairment, in addition to being observed in conditions of nutritional zinc deficiency (Yu et al, 2013), can be also obtained through zinc chelation in the hippocampus (Frederickson et al., 1990) or through genetic suppression of zinc transport into synaptic vesicles (Sindreu et al., 2011). Experiments of zinc supplementation gave less clear results possibly due to neurotoxicity of extra-synaptic zinc excess (Frederickson et al., 1989; Koh et al., 1996). Chronic zinc supplementation starting during gestation and lasting throughout adulthood resulted in impaired learning of spatial tasks in rats (Railey et al., 2010). However, zinc supplementation starting with gestation and continuing postnatally in juvenile life was reported to improve spatial memory (Piechal et al., 2012). Furthermore, zinc supplementation in adult rats prevented cognitive deficits consequent to traumatic brain injury (Cope et al., 2011). Hippocampal synaptic plasticity and spatial memory, which were compromised in a mouse model of depression, were rescued by zinc administration through nanoparticles (Xie et al., 2012). In mice, supplementation with high zinc dosage from weaning up to 3 months of age resulted in hippocampal regressive alterations and hippocampal-dependent memory impairment, likely due to the toxic effect of the treatment schedule adopted (Yang et al., 2013). In genetic mice models of Alzheimer's disease (AD), long-term zinc supplementation worsened disease-related memory impairment, an outcome also affecting wild-type animals (Linkous et al., 2009). These results may be explained by the hypothesis of zinc-mediated modification of cognitive activity (Takeda and Tamano, 2014), which implies that excessive hippocampal zinc signaling results in cognitive impairment, in particular under pathological/stressful conditions.

Notwithstanding several inconsistencies in the existing literature, possibly related to timing and dosage of zinc deficiency/supplementation, as well to the species considered (mouse *vs.* rat), evidence for a role of zinc in synaptic plasticity, learning and memory is convincing. Due to the multiplicity of protein-zinc interactions, unraveling those specifically related to cognition is a difficult task. In a recent study based on mice knockout for the zinc vesicular transporter ZnT3, the role of zinc in some cognitive tasks has been attributed to a presynaptic indirect effect on ERK signaling at mossy fiber terminals (Sindreu et al., 2011).

In the present research, we demonstrate in adult rats that four week-long zinc supplementation, approximately doubling the normal intake, impairs memory consolidation and facilitates forgetting of stressful experience in two hippocampal-dependent cognitive tasks, i.e. contextual fear conditioning and inhibitory avoidance. This effect is not accompanied by altered ERK signaling, but rather is associated with dysregulation of a key kinase for memory mechanisms, GSK-3ß, whose activity is known to be regulated by zinc. The relevance of these results for humans, in the light of the widespread use of zinc integrators and the possible role of zinc supplementation in post-traumatic stress disorders (PTSD) will be discussed.

2. Experimental procedures

2.1. Animals and treatments

Male Wistar rats from Harlan Laboratories, weighing 230-260 g at the beginning of experiments were kept in controlled temperature and humidity conditions with a light/dark cycle of 12 h, under veterinary surveillance for animal health and comfort. Experiments were carried on according to Italian and European Community Council guidelines for the use of laboratory animals and were approved by the Bioethical Committee of the University of Bologna (protocol n° 21-72-2012). Animals were fed *ad libitum* with the Harlan Teklad global diet 2018, which contains 70 mg zinc/kg and resulted in a mean zinc intake of ~5 mg/kg body weight/day. Rats subjected to zinc supplementation were provided with drinking water containing 150 mg/L $ZnSO_4 \times 7H_2O$. Based on water consumption, the additional intake of elemental zinc was ~4.5 mg/kg body weight/day, thus approximately doubling the intake of control rats. No differences in food and water intake were recorded between normally-fed and zinc-supplemented animals and weight gain was the same in the two groups.

In the first experiment, zinc supplementation was carried on for 4 weeks before training both normally-fed and zinc-supplemented animals and testing them, 24 h after training, in the contextual fear conditioning (CFC) and the inhibitory avoidance (IA) tests.

In the second experiment, animals were subjected to training for the same behavioral tasks, divided into a zinc-supplemented and a normally-fed group the day after

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