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Zinc Ameliorates Nogo-A Receptor and Osteocalcin Gene Expression in Memory-Sensitive Rat Hippocampus Impaired by Intracerebroventricular Injection of Streptozotocin

Haluk Gumus[1] · Saltuk Bugra Baltaci[2] · Omer Unal[3] · Elif Gulbahce-Mutlu[4] · Rasim Mogulkoc[2] · Abdulkerim Kasim Baltaci[2]

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Abstract

Metabolic dysfunction is a critical step in the etiopathogenesis of Alzheimer's disease. In this progressive neurological disorder, impaired zinc homeostasis has a key role that needs to be clarified. The aim of this study was to investigate the effect of zinc deficiency and administration on hippocampal Nogo-A receptor and osteocalcin gene expression in rats injected with intracerebroventricular streptozotocin (icv-STZ). Forty male Wistar rats were divided into 5 groups in equal numbers: Sham 1 group received icv artificial cerebrospinal fluid (aCSF); Sham 2 group received icv a CSF and i.p. saline; STZ group received 3 mg/kg icv STZ; STZ-Zn-deficient group received 3 mg/kg icv STZ and fed a zinc-deprived diet; STZ-Zn-supplemented group received 3 mg/kg icv STZ and i.p. zinc sulfate (5 mg/kg/day). Hippocampus tissue samples were taken following the cervical dislocation of the animals under general anesthesia. Nogo-A receptor and osteocalcin gene expression levels were determined by real-time-PCR method. Zinc supplementation attenuated the increase in hippocampal Nogo-A receptor gene expression, which was significantly increased in zinc deficiency. Again, zinc supplementation upregulated the intrinsic protective mechanisms of the brain by activating osteocalcin-expressing cells in the brain. The results of the study show that zinc has critical effects on Nogo-A receptor gene expression and hippocampal osteocalcin gene expression levels in the memory-sensitive rat hippocampus that is impaired by icv-STZ injection. These results are the first to examine the effect of zinc deficiency and supplementation on hippocampal Nogo-A receptor and osteocalcin gene expression in icv-STZ injection in rats.

Keywords Icv-STZ injection · Zinc · Hippocampus · Nogo-A receptor · Osteocalcin

Introduction

Alzheimer's disease is a neurodegenerative disease characterized by regression in cognitive functions and impaired memory ability [1]. The first pathology to appear in Alzheimer's is synaptic deterioration [2]. Nogo-A, which is a myelin protein encoded by the Nogo gene, is chemically composed of 1192 amino acids [1]. Nogo-A is abundantly expressed in both neurons and oligodendrocytes throughout the central nervous system, particularly at high levels in the olfactory bulb [2]. In addition, Nogo-A expression is also present in pyramidal cells and interneurons in the hippocampus, spinal motor neurons, and dorsal root ganglion cells [2]. The overexpression of Nogo-A, one of the nogo proteins known as Reticulon-4, which draws attention with its axonal growth inhibitory property, in the hippocampus of Alzheimer's patients has attracted attention [3]. For this reason, it is assumed that there may be a relationship between Nogo-A, which has a plasticity-suppressing effect, and Alzheimer's disease. Recent studies have already revealed evidence that Nogo-A factor plays a role in the pathogenesis of Alzheimer's disease [4]. It has also been reported that Nogo-A

✉ Haluk Gumus
hgumus@selcuk.edu.tr

1 Department of Neurology, Medical Faculty, Selçuk University, Konya 42031, Turkey

2 Department of Physiology, Medical Faculty, Selçuk University, Konya, Turkey

3 Departments of Physiology, Medical Faculty, Kirikkale University, Konya, Turkey

4 Department of Medical Biology, Medical Faculty, KTO Karatay University, Konya, Turkey

can affect hippocampal β-amyloid formation and tau levels through β-amyloid metabolism [5]. In particular, inhibition of Nogo protein has been shown to reduce β amyloid formation and improve cognitive performance in mice [6].

Osteocalcin is one of the osteocalcin receptor activations that can prevent the deterioration in cognitive functions through brain-derived neurotrophic factor (BDNF) [7–9] . [Oury et al. 10] showed that bone-derived osteocalcin not only mediates learning performance but also has a maternal effect on brain development during intrauterine life. The dentate gyrus (DG) acts as a gate that controls the input of information to the hippocampus, which is a critical structure in cognitive functions such as learning and memory. Therefore, DG plays a role in the regulation of cognitive behaviors [11]. Sun et al. [11] identified a subtype of nerve cells in the outer layer of dorsal dentate granule cells (dDGCs). This neuron group is specifically activated by anxiogenic stimuli and surprisingly shown to be expressing osteocalcin. The authors concluded that osteocalcin-expressing neurons in the adult hippocampus can suppress anxiety by promoting neurogenesis [11].

Zinc, which is indispensable in the regulation of many biological functions in living organism, is an important trace element [12]. In addition to acting as a cofactor for hundreds of metalloproteins or enzymes, zinc plays critical roles in a variety of events such as immune mechanisms, cell division, protein, and DNA synthesis [12]. Zinc dysregulation leads to deterioration in brain functions, leading to the onset of chronic pathologies including depression, schizophrenia, Alzheimer's, and aging [13]. In parallel, dietary zinc deficiency may increase the risk and progression of Alzheimer's [13]. Therefore, there is a proven relationship between zinc and Alzheimer's disease and zinc-Alzheimer's disease-cognitive functions [14].

The aim of this study was to investigate the effect of zinc deficiency and administration on hippocampal Nogo-A receptor and osteocalcin gene expression in rats injected with intracerebroventricular streptozotocin (icv-STZ).

Materials and Methods

Experimental Design, Animals, and Groups

The study was carried out in the Molecular Physiology Laboratory of Selçuk University Faculty of Medicine. Forty male Wistar rats, obtained from the Experimental Medicine Research and Application Center of Selçuk University, were used. (Male rats were preferred in the study so that the results of the study were not affected by the variations in hormonal parameters between the sexes). The study protocol was approved by the Ethics Committee of Selcuk University Experimental Medicine Research and Application Center (Decision No. 2019–44, dated 27.09.2019).

All animals were housed under a constant 12:12-h light–dark cycle and fed ad libitum. Animals were divided into 5 groups in equal numbers ($n=8$): Sham 1 group: icv received artificial cerebrospinal fluid (aCSF); Sham 2 group: received icv aCSF and i.p. saline; STZ group: the animals in this group were injected with 3 mg/kg/STZ as icv at 2-day intervals; STZ-zinc-deficient group: the animals in this group were injected with 3 mg/kg/STZ as icv at 2-day intervals. Animals in this group were fed a zinc deficient diet after surgical procedures. Animals of STZ-ZnDef group were fed a zinc-deficient diet containing 28 mg/kg zinc (Table 1), instead of 130 mg/kg zinc of standard diet.

STZ-zinc supplement group: The animals in this group were injected with 3 mg/kg/STZ as icv at 2-day intervals. Intraperitoneal (i.p) zinc sulfate (5 mg/kg/day) injection was given to the animals in this group after the surgical procedures. Rats i.p. 70 mg/kg of ketamine and 8 mg/kg of xylazine were placed in a stereotaxic frame (Small Animal Stereotaxic System, ASI Instruments, USA). STZ was dissolved in aCSF and injected into the animals at a dose of 3 mg/kg. Each animal was injected with 10 µL of STZ or aCSF into each ventricle (injection coordinates: 4.8 mm dorsoventral, 0.8 mm anteroposterior, and 1.4 mm lateral to bregma), for a total of 20 µL at 48-h intervals. After a 4-day adaptation period, icv injections were made on the 5th and 7th days.

Zinc sulfate was dissolved in saline and i.p. injected at a dose of 5 mg/kg to animals in the STZ-ZnSup group. Animals in the STZ-ZnDef group were fed a diet deficient in zinc [15]. Zinc-deficient rat food was purchased from Arden Research and Experiment (Ankara, Turkey). Intraperitoneal injections of zinc and dietary changes were started on day

Table 1 Content of some trace elements and minerals in zinc deficient rat diet

Content	Value	Unit
Aluminum	80.00	mg/kg
Iron	2.25	mg/kg
Iodine	1.66	mg/kg
Cobalt	0.34	mg/kg
Copper	5.00	mg/kg
Manganese	0.30	mg/kg
Molybdenum	100.00	mg/kg
Sulfur	1.10	mg/kg
Selenium	1.14	mg/kg
Zinc	28.20	mg/kg
Calcium	8.30	mg/kg
Potassium	7.95	mg/kg
Magnesium	2.55	mg/kg
Sodium	1.90	mg/kg
Phosphorus	5.76	mg/kg

7 and continued for 28 days (4 weeks) until the end of the experiments.

Twenty-four hours after the experimental stages of the study were completed, the life of all animals was terminated by cervical dislocation under general anesthesia, and hippocampus tissue samples were taken. (In order for the last zinc injection to be fully effective in the body, it was thought that it would be more appropriate to sacrifice the animals 24 h after the administration.) Hippocampus tissue samples were first taken into liquid nitrogen. Then, it was kept at − 80 °C until RT-PCR analysis. Osteocalcin and Nogo-A gene expression analyses were performed with RT-PCR technique in hippocampus tissue samples.

In our previously published study, it was determined that a sAH model was created in animals as a result of memory performance evaluated with the Morris water maze [15]. This study was performed on hippocampus tissues from the same animals.

RNA Isolation and RT-PCR Analysis

Total RNA from hippocampus tissue was isolated with a commercial RNA isolation kit (Bio Basic, Canada) according to the manufacturer's recommendation. After the purity and amount of the obtained RNA were determined, cDNA synthesis was started. cDNA using cDNA synthesis kit OneScript Plus (ABM, Canada) reverse transcriptase for mRNAs.

Primer sets used for expression analysis of target genes at the mRNA level are specific for each transcription analysis using FastStart Essential DNA Green Master Mix (Roche, Switzerland) following the manufacturer's instructions. The studied primers (Table 2) were synthesized in the manufacturing company (Biomers). For qPCR analysis, PCR was set up at 95 °C for 10 s, annealing at 60 °C for 10 s, and polymerization at 70 °C for 10 s, and the number of cycles was 45. Melting curve analysis was performed as 1 cycle, 15 s at 95 °C, 10 s at 60 °C, and 10 s at 95 °C. All reactions were performed on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA).

RT-PCR data analysis was performed using the $2^{-\Delta\Delta CT}$ method developed by Livak and Schmittgen [16].

Statistical Analysis

Statistical evaluation of the findings was made with the SPSS 22.0 package program. Arithmetic means and standard error of all parameters were calculated. The results were expressed as the mean ± standard error of the mean. In order to determine the homogeneity of the data, the "Shapiro–Wilk" test was performed, and it was determined that the data showed normal distribution. One-way analysis of variance test was used to determine the differences between groups, and Bonferroni test was used to determine the origin of the difference. Differences at the level of $p < 0.05$ were considered significant.

Results

Hippocampal Nogo-A Receptor Gene Expression Results

icv-STZ injection caused a significant increase in Nogo-A receptor gene expression compared to Sham groups ($p < 0.05$). The highest Nogo-A receptor gene expression was obtained with dietary zinc deficiency ($p < 0.05$). Nogo-A receptor gene expression of the STZ-zinc-supplemented group was higher than the Sham groups ($p < 0.05$) and lower than the STZ group ($p < 0.05$, Fig. 1).

Hippocampal Osteocalcin Gene Expression Results

icv-STZ injection caused a significant decrease in osteocalcin gene expression compared to Sham groups ($p < 0.05$). Dietary zinc deficiency did not cause a significant change when compared to the group that received icv-STZ injection. The highest osteocalcin values were obtained with zinc supplementation ($p < 0.05$, Fig. 2).

Discussion

Nogo-A is a protein that inhibits axonal regeneration, which is considered a major barrier to nerve regeneration after injury in mammals [1]. Recent reports show

Table 2 Primer sets used for qPCR reactions

Gene	Primer Sequence	Function
Osteocalcin	FW: AAGCCCAGCGACTCTGAGTCT	Target gene
	RW: GCTCCAAGTCCATTGTTGAGGTA	
Nogo-A Receptor	FW: ATCTTCCTGCACGGCAACCGAAT	Target gene
	RW: AGAGGTTGTTGGCAAACAGGTAG	
GAPDH	FW: GGGCCAAAAGGGTCATCATC	Reference gene
	RW: AACCTGGTCCTCAGTGTAGC	

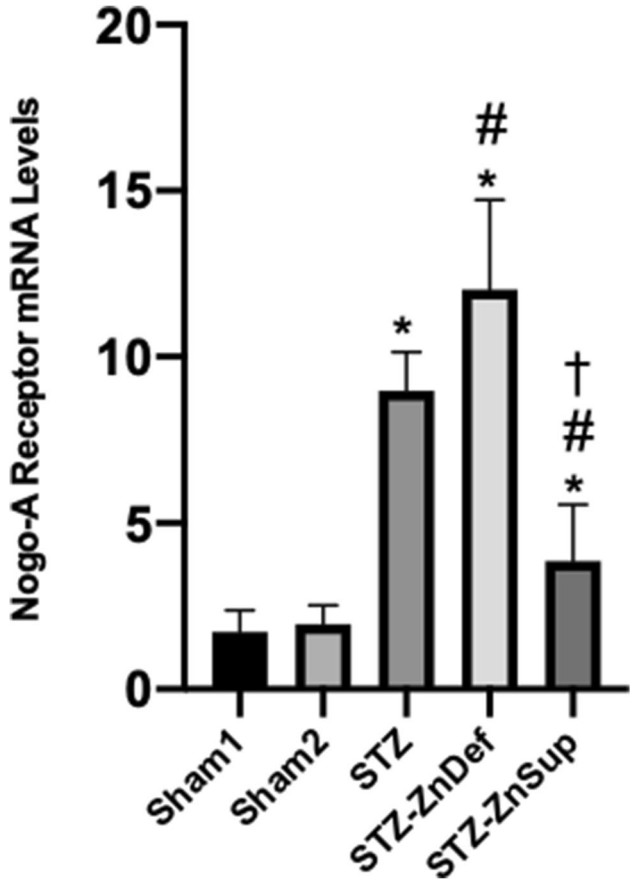

Fig. 1 Nogo-A receptor mRNA Levels. $*p<0.05$ versus Sham groups, $\#p<0.05$ versus STZ group, $\dagger p<0.05$ versus STZ-ZnDef group

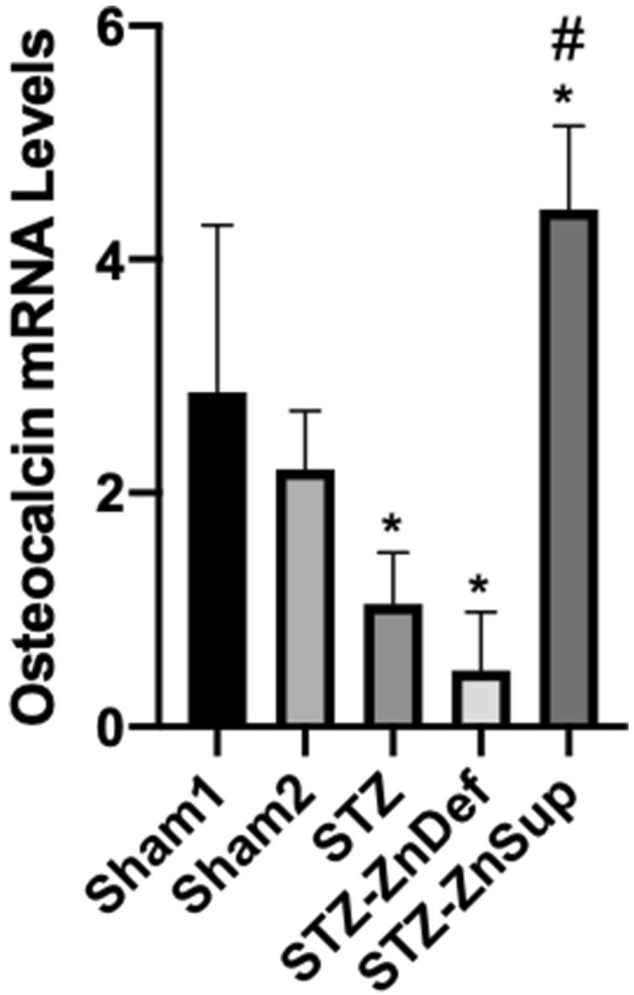

Fig. 2 Osteocalcin mRNA levels. $*p<0.05$ versus Sham groups, $\#p<0.05$ versus STZ and STZ-ZnDef groups

that important information has been obtained about the pathophysiological role of Nogo-A factor in Alzheimer's disease. It has been shown that Nogo-A plays a critical role in the molecular pathways that lead to the formation of Alzheimer's disease by binding to the Nogo-A receptor [1]. In particular, it has been found that Nogo-A/Nogo-A receptors modulate the formation of amyloid β-protein (Aβ), which is thought to be the main cause of Alzheimer's disease [1]. The gene expression of hippocampal Nogo-A in normal human aging and Alzheimer's disease was analyzed in detail by Gil et al. [17]. The results indicate that Nogo-A is expressed by oligodendrocytes and neurons in the aged hippocampal formation [17]. In the same study, it was reported that the overexpression of Nogo-A by hippocampal neurons with Alzheimer's is associated with beta-amyloid deposits in senile plaques [17]. The highest Nogo-A receptor gene expression levels in this study were obtained in the icv-STZ-zinc-deficient group. Nogo-A receptor gene expression values of the icv-STZ group were lower than the icv-STZ zinc-deficient group and higher than all other groups. This finding was similar to that of Gil et al. [17] who reported the overexpression of Nogo-A by Alzheimer's hippocampal neurons.

The most striking conclusion regarding the Nogo A findings of the present study is the inverse relationship between hippocampal Nogo-A expression and zinc. Nogo-A expression was higher in the icv-STZ zinc-deficient group than in the icv-STZ group. Zinc supplementation significantly decreased Nogo A gene expression in the icv-STZ group compared to the icv-STZ zinc deficient and icv-STZ group.

The opposite relationship between hippocampal Nogo-A expression and zinc, which we obtained in this study, is the first relevant finding.

The ability of the dentate gyrus (DG) to generate new neurons, also known as neurogenesis, is well known in rodents and non-human primates [18]. This ability to generate new neurons, which is seen in adults, has become a focus of attention, particularly in two areas. The first of these is their involvement in hippocampal molecular pathways, and the second is that they affect learning and memory

mechanisms. As a result, beyond regulating information processing, memory, and learning, hippocampal adult neurogenesis also stands out as a critical regulator of events such as depression [18]. Sun et al. [11] described a subtype of neurons in the outer layer of dorsal dentate granule cells that response specifically to anxiogenic stimuli and express osteocalcin. In the same study, the authors reported that activation of Ocn-expressing dDGCs suppressed anxiety-like behaviors and promoted adult DG neurogenesis, and both functions are likely mediated by brain-derived neurotrophic factor (BDNF) [11].

In this study, the highest osteocalcin gene expression levels were obtained in the icv-STZ zinc-supplemented group, and the lowest osteocalcin gene expression values were obtained in the icv-STZ and icv-STZ-zinc-deficient groups. Perhaps, the most interesting findings of the current study are hippocampal osteocalcin gene expression levels. In this study, we clearly demonstrated the relationship between icv-STZ injection, hippocampal osteocalcin gene expression, an important region in memory performance, and zinc.

Conclusion

The results of this study show that zinc has critical effects on the Nogo-A receptor and hippocampal osteocalcin gene expression levels in hippocampus tissue, a structure associated with cognitive performance. The present study is the first to examine the effect of zinc deficiency and supplementation on hippocampal Nogo-A receptor gene expression and osteocalcin gene expression.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval The study protocol was approved by the Experimental Animals Ethics Committee of Selcuk University Experimental Medicine Research and Application Center (2019–44).

Conflict of Interest The authors declare no competing interests.

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