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Syntaxin4 and Synapsin3 mRNA Levels Induced by Long-Term Iron Toxicity in Rat (*Rattus norvegicus*)

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ABSTRACT: Although iron is necessary for brain tissue, its overload causes many metabolic disorders as well as neurodegenerative disorders. Syntaxin4 and synapsin3 are proteins associated with synaptic vesicles that play a role in neurotransmitter release. In this study we investigated the effects of sublethal doses of ferric iron at 0.87 ppm, 3 ppm, 30 ppm and 300 ppm on mRNA levels of genes coding syntaxin4 and synapsin3 proteins that function in nerve cell signal transmission in cortex and hippocampus tissues of rats (*Rattus norvegicus*). While mRNA level of the syntaxin4 gene was unchanged in the cortex, mRNA level of syntaxin4 decreased with 30 ppm and 300 ppm exposure in the hippocampus. mRNA level of synapsin3 gene in both cortex and hippocampus tissues increased in all treatment groups compared to control group.

Key words: Iron overload, mRNA expression, syntaxin4, synapsin3

Sıçan (*Rattus norvegicus*)'da Uzun Süreli Demir Toksisitesi ile Uyarılan Sintaksin4 ve Sinapsin3 mRNA Seviyeleri

ÖZET: Demir beyin dokusu için gerekli olmasına rağmen aşırı birikimi nörodejeneratif rahatsızlıkların yanında birçok metabolik bozukluklara neden olmaktadır. Sintaksin4 ve sinapsin3 nörotransmitter salınımında rol oynayan sinaptik veziküller ile ilişkili proteinlerdir. Biz bu çalışmada ferrik demirin (Fe^{3+}) 0.87 ppm, 3 ppm, 30 ppm ve 300 ppm subletal dozlarına maruz kalmış sıçanların (*Rattus norvegicus*) korteks ve hipokampus dokularındaki sinir hücre sinyal iletiminde fonksiyonu olan Sintaksin4 ve Synapsin3 proteinlerini kodlayan genlerin mRNA seviyeleri üzerine etkilerini araştırdık. Sintaksin4 geninin mRNA seviyesi korteks dokusunda değişmez iken hipokampus dokusunda 30 ppm ve 300 ppm maruziyeti ile mRNA seviyesi azalmıştır. Hem korteks hem de hipokampus dokularında sinapsin3 geni mRNA seviyesi artmıştır.

Anahtar Kelimeler: Demir birikimi, mRNA ifadesi, sintaksin4, sinapsin3

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INTRODUCTION

Iron presence is necessary for many vital functions in many tissues besides brain tissue in all mammalian cells. These functions include high respiratory activity of brain tissue, myelinization, formation of GABAergic activity, and the production of many neurotransmitters such as dopamine, neuroprine, serotonin. Iron is a cofactor for enzymes involved in the synthesis of neurotransmitters such as tyrosine hydroxylase and tryptophan hydroxylase (Moos and Morgan, 2004). People are often exposed to iron via several industrial, agricultural, domestic and technological applications (Bradl, 2005). But when the iron concentration reaches uncontrollable levels, it is a source of concern for the brain because of its high reactivity. The inability of the brain cells to regenerate further exacerbates the toxicity of iron (Ponka, 2004). Regional iron accumulation at an abnormal level has been observed in the specific tissues of many dementia and neurodegenerative disorders including Alzheimer (Bishop et al., 2002), Huntington (Dexter et al., 1991) and Parkinson (Kaur and Andersen, 2004).

The synaptic conditions are mediated by signal events at the pre- and post-synaptic terminals of neurons. The release of neurotransmitters into the synaptic cleft is mediated by soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) that serve a function in membrane fusion. As a result, the v-SNAREs in the synaptic vesicle membrane are matched with the t-SNAREs in the presynaptic membrane via action potential. This complex performs the fusion by approximating the membranes (Harris et al., 2016). Syntaxins together with many protein families constitute the SNARE proteins. Syntaxins are a family of transmembrane proteins that fuse intracellular vesicles to target membranes. Furthermore, syntaxins also mediate and control the membrane composition (Jahn and Scheller, 2006; Martens and McMahon, 2008). Syntaxin4, one of this protein family, is required for various occurrences of membrane composition events such as membrane fusion in axon and postsynaptic regions (Harris, et al., 2016), myelin biogenesis in oligodendrocytes (Biljard, et al., 2015), exocytosis in astroglial plasma membranes (Tao-Cheng, et al., 2015) and membrane fusion in the cytoplasm (Chen and Scheller, 2001).

Synapsins are a family of neuron-specific phosphoproteins identified about 30 years ago (Johnson et al., 1972). Synapsin3 like other synapsins is also associated with synaptic vesicles that play a role in neurotransmitter release (Kao et al., 1998). In this context, they perform critical functions in binding of synaptic vesicles to the cytoskeletal networks of presynaptic terminals (Pieribone et al., 1995). The synapsins are phosphorylated via various kinases to alter affinities to actin filaments, thus it occurrences transferring the vesicles to the presynaptic terminal to induce signal transduction (Chi et al., 2001). It has been reported that synapsin3 regulates the size of the recycling pool of synaptic vesicles, neurotransmitter release (Kile et al., 2010), synaptic depression that limiting the exo- and endo-cytotic vesicles (Feng et al., 2002) and the inhibition of post-synaptic current, and plays also a role in neurodegenerative disorders (Stöber et al., 2000; Chen et al., 2009; Porton et al., 2010) due to its strong association with the release of dopamine (Kile et al., 2010).

In this study, we investigated the effects of ferric iron (Fe^{3+}) on mRNA levels of synapsin3 and syntaxin4 genes that play a role in synaptic transmission in neurons in rat cortex and hippocampus tissues. Thus we mainly aimed to contribute to determine these functions and to the literature about these genes.

MATERIALS and METHOD

Experimental animal and experimental application

Male Sprague Dawley (*Rattus norvegicus*) rats obtained from Atatürk University (Erzurum, TURKEY), Experimental Medical Application and Research Center were used in the study. The animals were kept under optimum conditions, with a temperature of 22 ±2 °C, 40-60 % humidity and a light-dark period of 12-12 s. Animals were also acclimatized with deionized water for a week before experimental application. In the following weeks the concentration was adjusted by mixing the waters with iron ($FeCl_3.6H_2O$) according to the concentrations of the groups. Rats were exposed to deionized water (DIW) mixed with daily metal for 100 days. In the experiment, 5 groups of rats were experimented. Concentrations are summarized in

Table 1. Rats were killed by anesthesia. During the experimental process, no animals died and no toxic signs were seen. Parts of the brain were quickly removed and frozen at -80 ºC for experimental steps. Animal experiments were carried out in accordance with the applicable national guidelines for the use and care of laboratory animals and were approved by the Local Animal Ethics Committee of Ataturk University.

Table 1. Concentrations of Fe^{3+} and feeding times given to rat groups

Week	Control	MPL 0.87 ppm	1X 3 ppm	10X 30 ppm	100X 300 ppm
1	DIW	DIW	DIW	DIW	DIW
2	DIW	MPL	MPL	MPL	MPL
3	DIW	MPL	1X	1X	1X
4	DIW	MPL	1X	10X	10X
5	DIW	MPL	1X	10X	100X
6	DIW	MPL	1X	10X	100X
7	DIW	MPL	1X	10X	100X
8	DIW	MPL	1X	10X	100X
9	DIW	MPL	1X	10X	100X
10	DIW	MPL	1X	10X	100X
11	DIW	MPL	1X	10X	100X
12	cutting	MPL	1X	10X	100X
13		cutting	1X	10X	100X
14			cutting	10X	100X
15				cutting	100X
16					cutting

MPL: Maximum Permissible Limit, ppm: Parts per million

RNA extraction and cDNA synthesis

Total RNA was manually isolated from 500 mg of frozen tissues by using TRIzol reagent (Invitrogen). RNA concentrations and quality were verified by means of spectrophotometer (NanoDrop) and RNA gel electrophoresis, respectively. Afterwards, RNA samples were stored at -80 °C. cDNA synthesis was performed with SuperScript® First-Strand Synthesis System (Invitrogen) kit using 4 µl total RNA. After the quantification of the single-stranded cDNA was synthesized by the nanodrop (Thermo), the correctness of the synthesis using classical PCR has been proven. Samples were stored at -20 ºC until working.

TaqMan probe and primer design

Synapsin3, Syntaxin4 and Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) primers and probes belonging to Rattus norvegicus were designed using primer design program (http://primer3.ut.ee/). The specific region specificities of the oligonucleotide sequences obtained were checked by blasting in GenBank. The oligonucleotide sequences, product lengths and genbank accession numbers belonging to primer and probes used are given in Table 2.

Table 2. Genes specific forward and reverse primers, TaqMan probes, product lengths and genebank accession numbers

Gene	Primers and Probes	Sequence (5'→3')	Product Length	Genebank Accession Numbers
Syntaxin4	Forward	5'-TGCCATCTGTGTTTCTGTCAC-3'	114	NM_031125
	Reverse	5'-AAATGCTGGGTCTCTGTTTCTC-3'		
	Probe	5'-$^{FAM-}$ TGGCTGTCATCATTGGCATCACC $^{-TAMRA}$-3'		
Synapsin3	Forward	5'-AGACAGACAAGGCATCACTACATC-3'	115	NM_017109
	Reverse	5'-AGCCCAAAGGCAAGCAAAAT-3'		
	Probe	5'-$^{FAM-}$ ACCATACGCTTGCTCCCTGCCG^{-TAMRA}-3'		
Gapdh	Forward	5'-TGGACCTCATGGCCTACATG-3'	144	NM_017008.4
	Reverse	5'-AGGGAGATGCTCAGTGTTGG-3'		
	Probe	5'-$^{CY5-}$ ACCACCCAGCCCAGCAAGGA $^{-BHQ2}$-3'		

Gene expression analysis

Quantitative determination of gene expression by Real Time PCR was performed with Rotor-Gene Q (Qiagen). PCR was carried out as multiplex in 50 µl volumes containing 900 nM forward and reverse primers, 250 nM TaqMan probes and 25 µl FastStart TaqMan Probe Master (Applied Biosystems) that contains in AmpliTaq Gold DNA Polymerase, AmpErase uracil N-Glycosylase (UNG), dNTP with dUTP optimize buffer component.

The thermal conditions for amplification and determination of the samples and standards are as follows; 2 min at 50 ºC, 10 min at 95 ºC, afterwards 45 cycles of 10 sec at 95 ºC, and 1 min at 60 ºC. The data were analyzed using method (e)$^{(-\Delta\Delta Ct)}$. Analytical precision was verified by considering the standard curve. The efficiency value is calculated from the slope curve using the formula (Pfaffl, 2001) and the slope value is known through Qiagen Rotor-Gene Q software.

Statistical analysis

All statistics were analyzed by ANOVA method and variance analysis in SPSS 17.0 package program. Duncan multiple comparison test was used to determine the significance difference among groups means as a result of ANOVA test and variance analysis.

The value of p less than 0.05 was considered statistically significant. All values are shown as Mean±S.D. (Mean±Standard Deviation). Each group contains three animals, and all measurements were triplicated for each animal.

RESULTS

The effects of iron (Fe^{3+}) on mRNA levels of genes encoding proteins involved in nerve cell signal transduction in cortex and hippocampus tissues of rats (*Rattus norvegicus*) exposed to subletal doses of 0.87 ppm, 3 ppm, 30 ppm and 300 ppm were investigated.

It was observed that the expression levels of syntaxin4 gene increased gradually, as it increased the ion concentration in the cortex tissue. However, the differences in mRNA levels among the all treatments (0.87 ppm, 3 ppm, 30 ppm and 300 ppm) were statistically insignificant when compared to controls and to each other ($p>0.05$) (Figure 1).

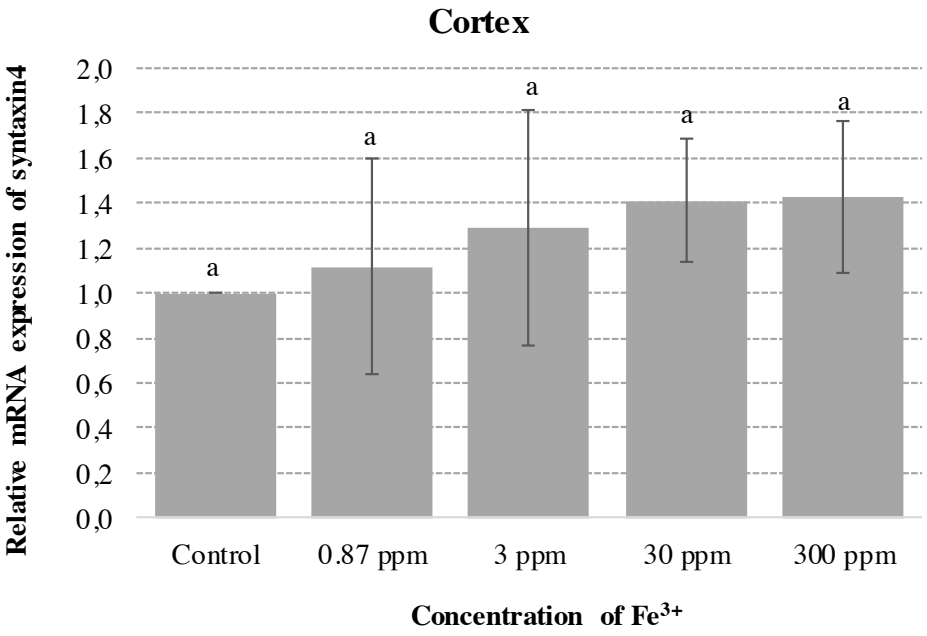

Figure 1. *In vivo* effect of various iron concentrations on the syntaxin4 gene in the rat cortex. a, b, c, d: statistical signifigance between each values in the lines. $p<0.05$ was considered as significant.

Hippocampal syntaxin4 mRNA levels were not significantly different between 0.87 ppm and 3 ppm groups when compared to both controls and each other ($p>0.05$). In the 30 ppm and 300 ppm groups, the mRNA levels were significantly reduced compared to the control, 0.87 ppm and 3 ppm groups ($p<0.05$), but the mRNA level difference between each others was not statistically significant ($p>0.05$) (Figure 2).

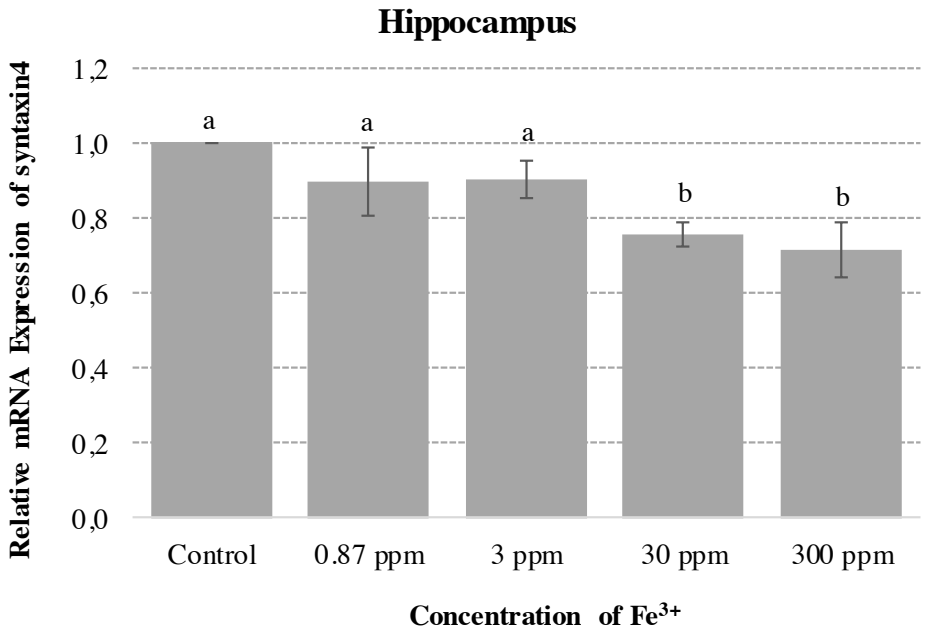

Figure 2. *In vivo* effect of various iron concentrations on the syntaxin4 gene in the rat hipoocampus. a, b, c, d: statistical signifigance between each values in the lines. $p<0.05$ was considered as significant.

When mRNA levels of synapsin3 in the cortex were experimented, no statistical differences was found in the 0.87 ppm group compared to the control group ($p>0.05$). A significant increase was observed in the 3 ppm group compared to the control group and 0.87 ppm group ($p<0.05$). The highest mRNA level was observed in the 30 ppm treatment and this increase of mRNA level was found to be significant compared to other groups ($p<0.05$). The mRNA level of the 300 ppm group was significantly decreased compared to the 30 ppm group and decreased to look like mRNA level of control group ($p<0.05$) (Figure 3).

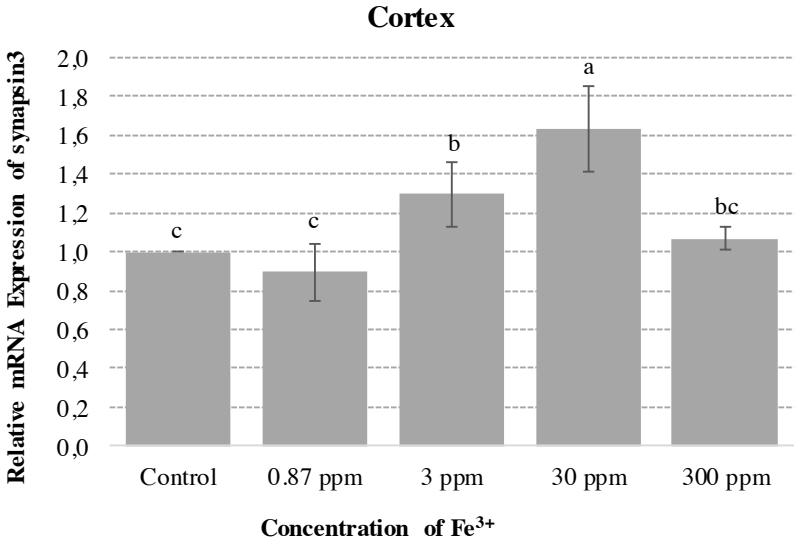

Figure 3. *In vivo* effect of various iron concentrations on the synapsin3 gene in the rat cortex. a, b, c, d: statistical signifigance between each values in the lines. $p<0.05$ was considered as significant.

When hippocampal Synapsin3 gene was considered, mRNA levels of the all treatments (0.87 ppm, 3 ppm, 30 ppm and 300 ppm) increased significantly compared to the mRNA level of the control group ($p <0,05$). The mRNA level differences among treatments were not statistically significant ($p>0.05$) (Figure 4).

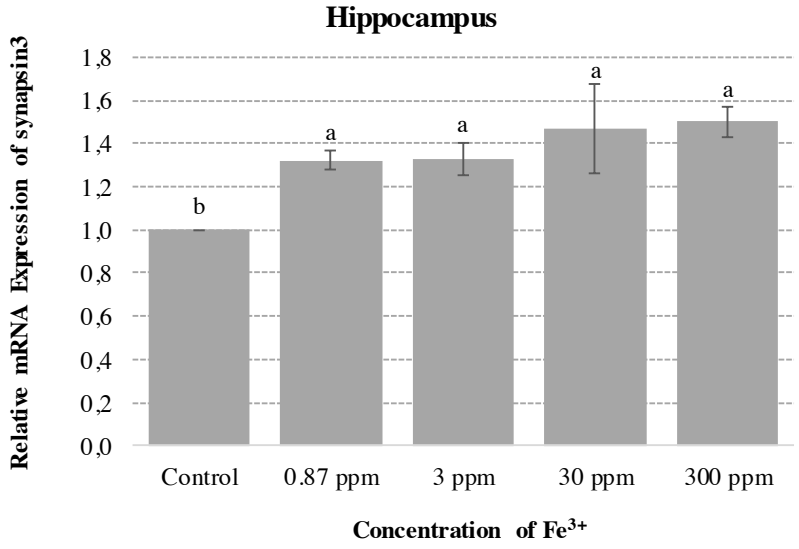

Figure 4. *In vivo* effect of various iron concentrations on the synapsin3 gene in the rat hippocampus. a, b, c, d: statistical signifigance between each values in the lines. $p<0.05$ was considered as significant.

DISCUSSION

It is known that iron is clearly require in neuron. Iron overload causes neuronal disorders. This is thought to be caused by affecting motor and cognitive functions as a result of impairment of energy metabolism and neurotransmitter synthesis (Moos and Morgan, 2004). Levels of neurotransmitter synthesis can affect the amount of synaptic vesicle and therefore, it can be expected to affect the mRNA levels of proteins that perform to formation of SNARE complex. Docking and fusion of synaptic vesicles to the plasma membrane are required for action potential in nerve cells. Syx4 is associated with membrane fusion in both axonic and postsynaptic regions (Harris et al., 2016). In this work, as the iron concentration increased, mRNA level of hippocampal Syx4 decreased. This may be for the reason that the accumulation of iron leads to a decrease in synaptic vesicle amount and as a result, it may decrease the action potential. Indeed, some metals are a potent inhibitors for action potential (Cooper et al., 1983) and this supports our hypothesis. However, in this study, there was no change in Syx4 mRNA level in the cortex.

Experiments have shown that in synapsin3-deficient neurons, a decrease in inhibitory post-synaptic potential occurs although the synaptic vesicle reserve does not change (Feng et al., 2002). This suggests that synapsin3 acts as a negative regulator of neurotransmission. It is known that synapsin3 protein is involved in slow synaptic transmission as it helps dopamine release as well as neuronal growth (Porton et al., 2011). In our study, the increase of synapsin3 mRNA level in the tissues means that synaptic transmission in rats is attempted to be inhibited by iron ions. Thus, the idea that iron ion can inhibits neurotransmission may arise. This idea also overlaps with the conclusion, we reveal from the syntaxin4 mRNA level. We guess that decreasing in mRNA level of 300 ppm treatment of the cortical synapsin3 may be induced by negative feedback regulation of the iron (Meyron-Holtz et al., 2004; Hentze et al., 2004).

CONCLUSIONS

Expression schemas in both cortex and hippocampus tissues are partially collateral with eachother. Our results have shown that iron overload can abnormally affect synaptic transmission mechanisms in neurons.

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