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PII: S1734-1140(16)30343-7
DOI: http://dx.doi.org/doi:10.1016/j.pharep.2017.01.008
Reference: PHAREP 622

To appear in:

Received date: 7-11-2016
Accepted date: 11-1-2017

Please cite this article as: Bernadeta Szewczyk, Katarzyna Kotarska, Agata Siwek, Łukasz Olech, Katarzyna Kuter, Antidepressant activity of zinc: further evidence for the involvement of the serotonergic system, http://dx.doi.org/10.1016/j.pharep.2017.01.008

Short Communication

Antidepressant activity of zinc: further evidence for the involvement of the serotonergic system

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Abstract

Background: The present study sought to further evaluate the role of the serotonergic system especially the postsynaptic 5-HT1A receptors (5-HT1AR) in the mechanism of antidepressant action of zinc.

Methods: Messenger RNA (mRNA), protein level, and 5-HT1AR density as well as the rate of monoamine (dopamine, DA, and serotonin) metabolism in the prefrontal cortex (PFC) and hippocampus (Hp) of rats subjected to acute and chronic (21 days) zinc (5mg Zn/kg) treatment were measured.

Results: Acute or chronic zinc treatment did not induce any changes in 5-HT1AR mRNA levels in the PFC or Hp of rats. However, chronic zinc treatment induced increases in both 5-HT1AR protein levels and density of 5-HT1A receptor binding sites in the Hp of rats. Chronic

zinc treatment also increased tissue levels of serotonin metabolite and turnover in the rat Hp. On the other hand, DA, DOPAC, HVA tissue levels increased while DOPAC/DA and 3MT/DA decreased in the PFC of rats after chronic zinc treatment. Acute treatment induced increases only in tissue levels of DOPAC, and DOPAC/DA.

Conclusions: Our results confirm that the antidepressant effects of zinc are mediated in concert with the modulation of the serotonergic system including postsynaptic 5-HT1ARs and allude to a possible involvement of dopaminergic neurotransmission in this action.

Keywords: zinc, 5-HT1ARs, dopamine, serotonin

Introduction

Zinc is thought to be involved in the pathophysiology and treatment of depression. Recent studies showed significant antidepressant-like effects of zinc after acute and chronic administration in several tests and models of depression (for review see [1]). Zinc's antidepressant activity is thought to be associated with the modulation of NMDA/glutamate-mediated neurotransmission (for review see [2]). The role of serotonergic neurotransmission in this action has also been suggested. For instance, the additive effects of zinc and selective serotonin reuptake inhibitors (citalopram and fluoxetine) in the forced swim test (FST) have been demonstrated [3, 4]. Moreover, the depletion of serotonin by para-chlorophenylanine (PCPA) completely blocks the antidepressant-like effects of zinc in the FST [5], indicating a requirement for an intact serotonergic system for these effects to occur. Additionally, earlier

studies, using a selective 5-HT1AR antagonist (WAY 100635) demonstrated the important role of these receptors in the antidepressant action of zinc in the FST [5, 6]. Recently, *in vitro* studies [6] showed the direct modulatory effects of zinc at the 5-HT1AR and indicated a concentration-dependent dual mechanism of zinc action at 5-HT1ARs, with potentiation at a low dose and inhibition at a high dose. The *in vivo* studies further show that zinc can modulate both presynaptic and postsynaptic 5-HT1ARs [6].

Based on the above data and the fact that adaptive changes in the serotonergic system are generally believed to underlie the therapeutic effect of antidepressant drugs, the present study was designed to further evaluate the role of zinc in the modulation of the serotonergic system using neurochemical, biochemical and radioligand binding assays.

Material and methods

Animals

Experiments were carried out with male Sprague-Dawley rats, (Charles, River, Germany), kept under standard laboratory conditions of lighting (light phase: 7:00-19:00) and temperature (19-21°C), with free access to water and food. All procedures were performed according to the guidelines of the National Institutes of Health Animal Care and Use Committee with approval from the Ethics Committee of the Institute of Pharmacology PAS in Krakow. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drug administration and tissue collection

Zinc (dose refer to mg Zn/kg) was administered intraperitoneally (*ip*) as zinc hydroaspartate (Farmapol, Poland) either acutely (30 min before decapitation) or chronically for 21 days (last dose administered 24h before decapitation). Controls were treated with 0.9% NaCl and are indicated as Veh on the graphs. Animals were sacrificed under non-stress conditions by rapid

decapitation following which brains were rapidly removed. Both the PFC and Hp were dissected on an ice-cold glass plate, frozen on dry ice and stored at -80°C until required.

Western blot analyses

Tissue preparation and western blot analyses were done as previously described [7]. Briefly, tissue samples from the PFC and Hp were homogenized in 2% solution of sodium dodecyl sulphate (SDS), denatured at 95°C for 10 minutes and finally centrifuged for 5 minutes at 10,000 rpm at 4°C. Proteins present in the supernatant were fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Invitrogen, Paisley, UK). Membranes were subsequently subjected to: blocking with 1% blocking solution (Roche), incubation (overnight at 4°C) with rabbit polyclonal anti-5-HT1AR antibody (1:1000, Abcam), washing (3x10 min) with Tris-buffered saline containing Tween 20 (TBS-T), incubation (30 min) with secondary anti-rabbit- IgG-peroxidase conjugated antibody (1:7000, Roche), washing (3x10 min) with TBS-T; incubation with detection reagent (Roche). To check for transfer and loading, β-actin was indicated on each blot (Millipore; 1:8000). The optical densities of the ensuing proteins were measured and analyzed using the Image Gauge v.4.0 software. Results are given as the ratio of the optical density of 5-HT1AR proteins to an optical density of β-actin and analyzed by Student t-test.

RNA isolation and real-time RT-PCR

The methods for RNA isolation and real-time RT-PCR have been described in detail in previous work [7]. Briefly, total RNA was extracted from homogenized tissue samples with TRIzol reagent (Invitrogen). The RNA integrity was evaluated by gel electrophoresis, while its purity and concentration were assessed using a Nanodrop spectrophotometer (Thermo Scientific). One microgram of total RNA of each sample was digested with DNase I (Sigma-Aldrich) and then reverse transcribed to complementary DNA (cDNA) using a High Capacity

cDNA Reverse Transcription Kit (random primers; Applied Biosystems). Real-time Polymerase Chain Reactions (PCRs) were performed in triplicates in a final volume of 18µl on a CFX96 Real-Time System (Bio-Rad) using Power SYBR Green Master Mix (Applied Biosystems) and primers specific for *5HT1A* (*Htr1a*) (forward: 5'-atcatgggcaccttcatcctctg-3', reverse: 5'-gctttcacagaaaggcaggaccag-3') or *Gapdh* (forward: 5'-agccgcatcttcttgtgcagtg-3', reverse: 5'-tggtaaccaggcgtccgatacg-3') at concentration of 200 nM each. Standard cycling conditions were applied: polymerase activation (95^{o}C, 10 min) and 40 PCR cycles (denaturation: 95°C, 15 s; annealing-elongation: 60^{o}C, 1 min) followed by melt curve analysis (at ramp +0.5°C). The specificity of each primer set was confirmed by checking melting temperature and the product size by gel electrophoresis. Results obtained for all samples were normalized to the loading control Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) - ΔCt values. Next, relative mRNA level indexes of *5HT1A*-were generated with the $2^{-\Delta Ct}$ formulae [8]. Statistical analyses of mRNA levels were performed on $2^{-\Delta Ct}$ values using the Student t-test.

HPLC-EC analysis of dopamine, serotonin concentrations and their metabolites in brain tissues

The concentrations of serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) as well as dopamine (DA) and its metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), homovanillic acid (HVA) were assessed using HPLC with electrochemical detection as published previously [9]. Tissue samples were weighed and homogenized in 0.1 M perchloric acid with 0.05 mM ascorbic acid and injected into the HPLC system (30°C, Hypersil Gold C18, 100 × 3.0 mm, 3 µm, Thermo Scientific). The mobile phase was composed of 50 mM NaH2PO4 × 2H2O; 40 mM citric acid; 0.25 mM 1-octanesulfonic acid sodium salt; 0.25 mM EDTA; 1.3% acetonitrile; 2.4% methanol. The

applied potential of the electrochemical detector was E1 = 350 mV and E2 = -220 mV. The resulting data were quantified using the area under the peaks and external standards with Chromeleon software (Dionex). The turnover rates were calculated as metabolite to neurotransmitter ratio. Results are presented as ng/mg wet tissue. Statistical analyses were performed using Student t-test.

5-HT1A binding assay

[3H]-8-OH-DPAT, [Propyl-2,3-ring-1,2,3-3H], spec. act. 135,2 Ci/mmol, 1mCi/ml, (PerkinElmer) was used for labeling 5-HT1ARs. Saturation binding studies were performed on rat hippocampal membranes as previously described [10]. Briefly, Hp was homogenized in 20 volumes of 50 mM Tris–HCl buffer (pH 7.7 at 25ºC) and centrifuged at 10,000 × g for 10 min The resulting pellet was resuspended in the same quantity of buffer, preincubated at 37°C for 10 min and centrifuged for 10 min again. The final pellet was resuspended in Tris–HCl buffer containing 10 µM pargyline, 4 mM CaCl2, and 0.1% ascorbic acid. Samples contained 400 µl of the tissue suspension (5 mg of wet weight), 50 µl of [3H]8-OH-DPAT solution (six concentrations ranging from 0.4 to 14 nM) and 50 µl Tris–HCl buffer. Nonspecific binding was determined in the presence of 10 µM serotonin. The reaction mix was incubated at 37 °C for 15 min and then filtered immediately onto GF/B glass fiber filter using 96-well FilterMate Harvester (PerkinElmer). The radioactivity retained on the filter was counted in a MicroBeta TriLux scintillation counter. Data were analyzed using interactive curve fitting routines (GraphPad Prism, Version 3.0, San Diego, CA). Statistical analyses were performed using Student t-test.

Results and Discussion

Our present study represents a further pharmacological evaluation of zinc effects at 5-HT1AR and serotonin neurotransmission in the context of zinc antidepressant-like activity. Since the

role of postsynaptic 5-HT1AR in the pathophysiology of depression and in the action of antidepressants is widely discussed in the literature (see [11] for review), our present study focused on the effect of zinc at postsynaptic 5-HT1AR in the PFC and Hp of rats after acute and chronic zinc treatments. Neither acute nor chronic zinc treatments induced significant changes in 5-HT1AR mRNA in the PFC or Hp (Fig. 1a, c). However, chronic zinc treatment increased protein levels of 5-HT1AR in the Hp by 78% (t=3.137, df=14, p=0.007) relative to controls (Fig. 1d).

Discrepancies between 5-HT1AR mRNA and protein expression levels in different brain regions have been reported in animal stress models. Iyo et al., observed increased 5-HT1AR mRNA and decreased protein levels in the PFC [12]. Earlier animal studies showed no changes in 5-HT1AR mRNA in the PFC of female rats subjected to prenatal stress, in pregnant stressed female or in the Hp of male rats subjected to chronic mild stress [7]. However, 5-HT1AR protein levels were reduced in these groups [7]. Shishkina et al., [13] reported an increase in 5-HT1AR gene expression in the PFC of rats exposed to FST; this effect was reversed by chronic fluoxetine treatment [13]. However, in the non-stressed rats, fluoxetine decreased the mRNA levels of 5-HT1A in the PFC but had no effect on these receptors in the Hp [13]. In the same study, the protein levels of 5-HT1AR increased in the Hp with no change in mRNA levels. Although, we did not use any stressful conditions in our studies, discrepancies between mRNA and protein levels of 5-HT1AR may not be uncommon in naïve rats and those treated with zinc.

In the receptor binding studies we found that chronic (Fig. 2b; t=2.401, df=10, p=0.037) but not acute (Fig. 2a) zinc treatment increases density of 5-HT1A binding sites in the Hp. Similar effects were observed earlier using a higher dose of zinc (11.3 mg Zn/kg) but for a shorter time (14 days) [14]. The present data thus confirmed previous findings on the

zinc-induced increase in 5-HT1AR density and further indicates that this effect is specific for chronic zinc treatment.

One of the effects induced by antidepressants is alterations in the monoamine levels and neurotransmission in the brain [15]. Some authors have assumed that high levels of 5-HIAA reflect elevated turnover rates of 5-HT and increased serotonergic neuronal activity (see [16] for review). Our present studies showed no changes in 5-HT, 5-HIAA levels or 5-HIAA/5-HT ratio after acute or chronic zinc treatment in the PFC (Fig.3a-c). However, significant increases in the level of 5-HIAA (by 23%) and in the 5-HT turnover rate (5-HIAA/5-HT, by 29%) [(t=2.91, df=11, p=0.014 and t=2.296, df=11, p=0.04 respectively] were found in the Hp after chronic zinc treatment (Fig. 3 e-f) indicating that zinc may enhance serotonergic neurotransmission in this brain structure and/or serotonin release.

In addition to serotonin and its metabolites we also measured the levels of DA and DA metabolites since the role of this monoamine in the etiology and treatment of depression has also been suggested [17]. In the PFC we found elevated levels of the DA metabolite DOPAC (by 45% ; t=783, df=10, p=0.019) with increase in DA turnover rate (DOPAC/DA) of 38% (t=2.3, df=10, p=0.044) after acute zinc treatment (Fig. 4 b, e). After chronic zinc treatment DA levels increased by 280% (t=2.455, df=9, p=0.03) and its metabolites DOPAC by 117% (t=3.164, df=9, p=0.01) and HVA by 98% (t=4.814, df=11, p=0.0005) although DA turnover rates were decreased (DOPAC/DA by 30%,t=2.474, df=9, p=0.03 and 3-MT/DA by 68%, t=3.532, df=11, p=0.004) (Fig. 4a-g). Surprisingly, no changes in DA, its metabolites and DA turnover rates were found in the Hp of rats after both acute and chronic zinc treatments (Fig. 4h-n).

The increased tissue content of DA in the PFC after chronic zinc treatment might reflect either elevated synthesis or weakened elimination. In the case of zinc, it is unlikely that a reduction in the elimination of DA could be due to a decreased activity of monoamine

oxidase since HVA and DOPAC levels were also elevated. However, decreased elimination might result from a decreased DA release since we found lower DA turnover ([DOPAC]/[DA] ratios) in the PFC of rats after chronic treatment. Seeing that decreased DA release may enhance synthesis via a compensatory feedback mechanism (see [15]), it is possible that zinc can also regulate DA synthesis. Interestingly, this effect seems to be brain region specific since no changes in the DA level or its metabolite were seen in the Hp.

In summary, the present findings provide additional evidence for the involvement of serotonergic neurotransmission in the mechanism of antidepressant activity of zinc and further confirm that zinc can be a potent modulator of postsynaptic 5-HT1ARs, a hallmark of clinically effective antidepressant drugs. On the other hand, the findings also indicate a role for DA neurotransmission in the action of zinc. However, this hypothesis will require further detailed evaluation.

Acknowledgements

This study was supported by a grant 2013/08/M/NZ7/00518 (B.S.) from the National Science Centre and partially by statutory funds of the Institute of Pharmacology, Polish Academy of Sciences.

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Figure legends

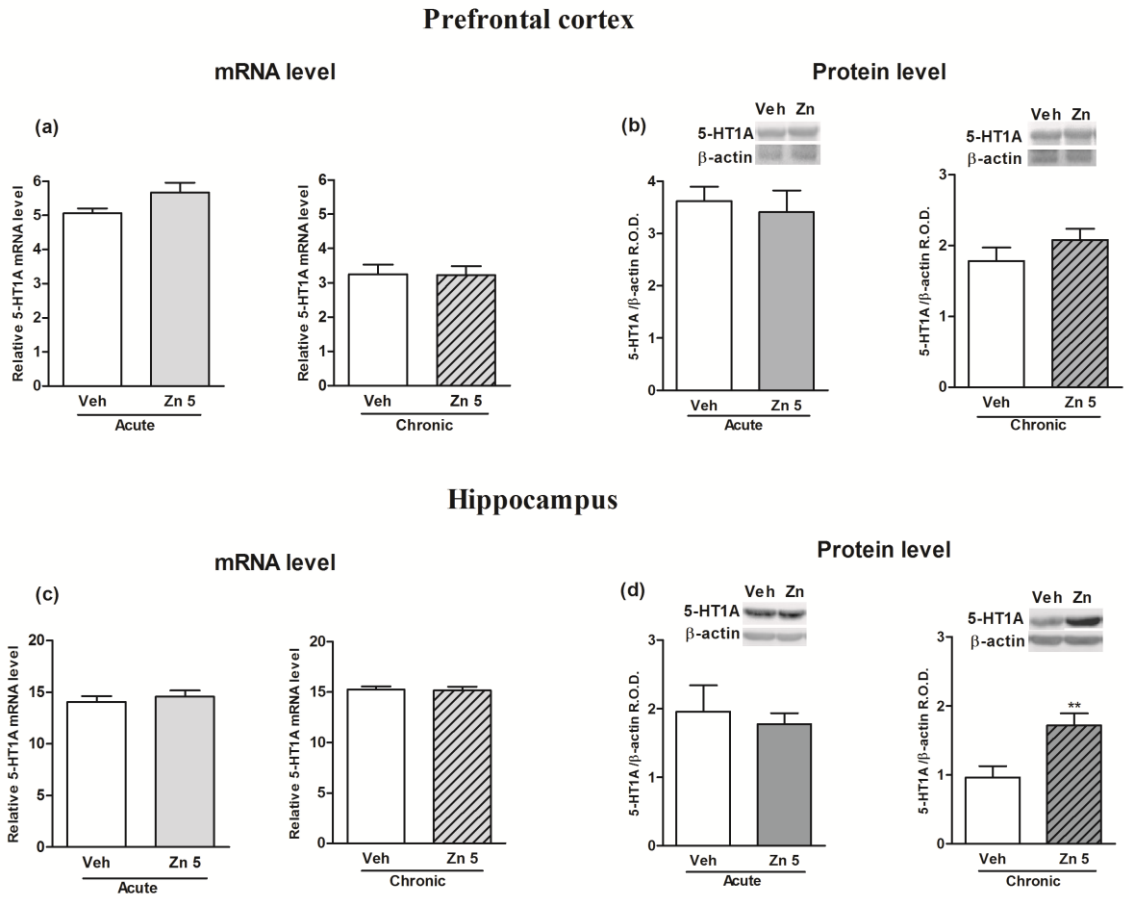

Figure 1. Analysis of mRNA and protein levels of 5-HT1AR in the PFC (**a, b**) and Hp (**c, d**) of rats subjected to the acute and chronic zinc treatments. Data are expressed as mRNA of 5-HT1A in relation to Gapdh ($2^{-\Delta Ct} \times 10^3$ values ± SEM from 7-9 samples) and as protein level of 5-HT1AR in relation to β-actin (ROD values ± SEM from 5-7 samples). The data were analyzed by Student t-test. *$p < 0.05$; ***$p < 0.001$ *vs.* Veh

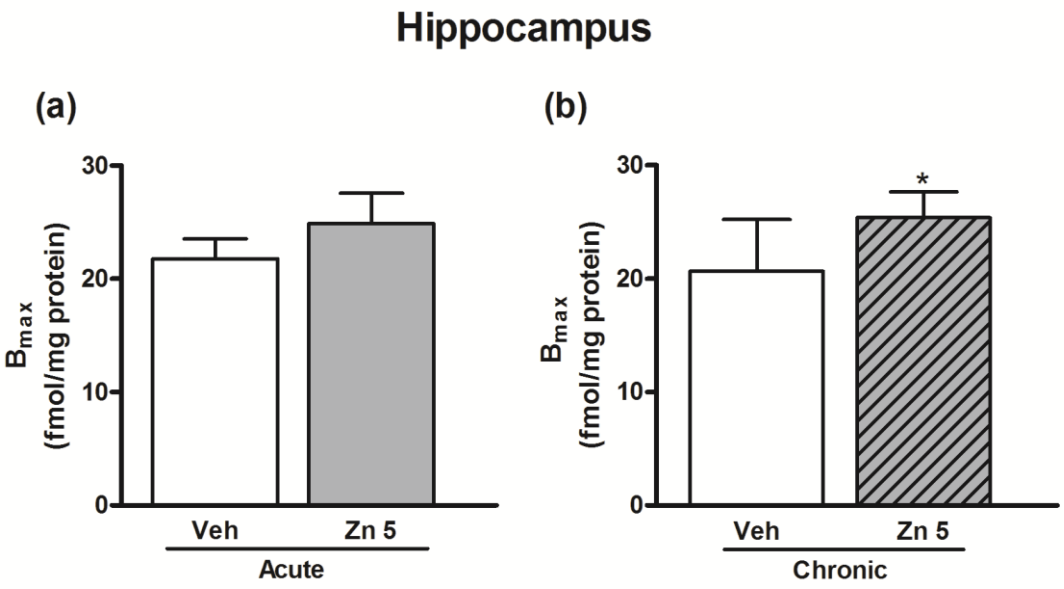

Figure 2. The effect of acute (**a**) and chronic (**b**) zinc administration on the density of [^{3}H]8-OH-DPAT binding to 5-HT1ARs in the rat Hp. The results are expressed as means ± SEM from 5-7 samples. The data were analyzed by Student t-test. *$p < 0.05$; ***$p < 0.001$ *vs.* Veh.

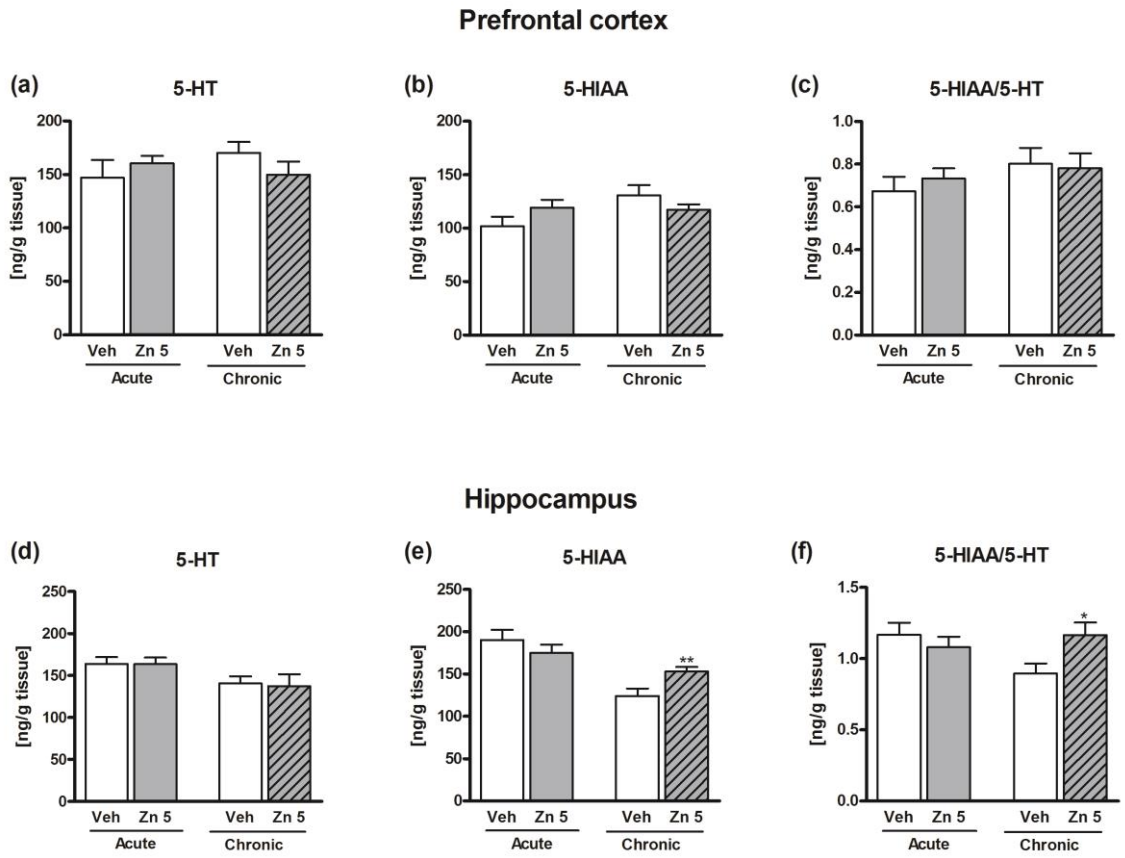

Figure 3. The effect of acute and chronic zinc treatments on tissue levels of 5-HT, its metabolite (5-HIAA) and turnover rates in the rat PFC (**a-c**) and Hp (**d-f**). Data are shown as means ± SEM, n=6-7 and were analyzed using Student's t-test. *$p < 0.05$, **$p < 0.001$ *vs.* Veh

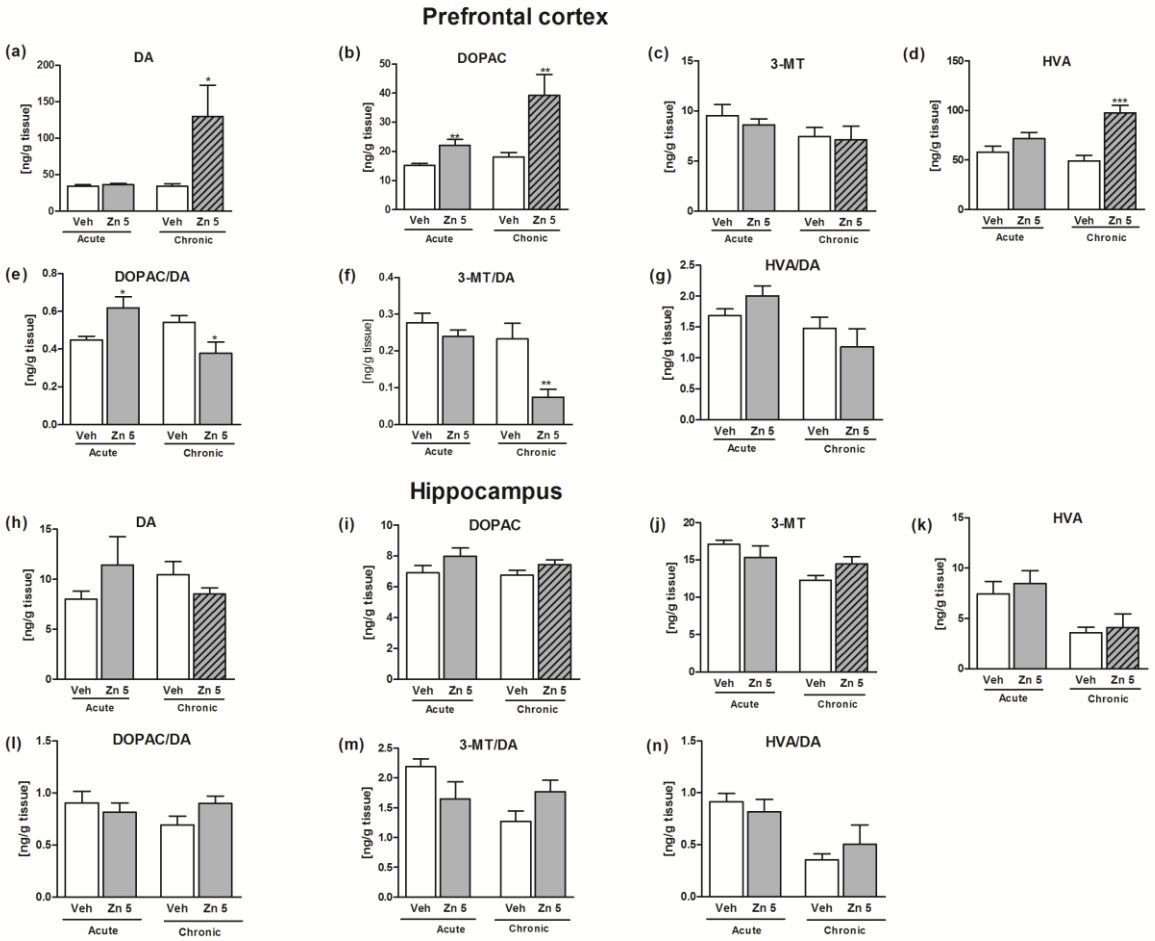

Figure 4. The effect of acute and chronic zinc treatments (5 mg/kg) on tissue levels of DA, its metabolites DOPAC, 3-MT, HVA and turnover in the rat PFC (**a-g**) and Hp (**h-n**). Data are shown as mean ± SEM, n=6-7 and were analyzed using Student's t-test. *$p < 0.05$, **$p < 0.001$ *vs.* Veh