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INTRAPERITONEAL ZINC ADMINISTRATION INCREASES EXTRACELLULAR ZINC IN THE RAT PREFRONTAL CORTEX

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Single administration of zinc evokes pharmacological behavioral effects in rodents, while no brain zinc alterations were detected. The aim of the present study was to examine the effect of a single zinc hydroaspartate intraperitoneal (*ip*) administration on the extracellular (synaptic) zinc concentration in the rat prefrontal cortex. We used anodic stripping voltammetric (ASV) method of zinc determination in microdialysate, which assays the extracellular zinc concentration. We report that acute (65 mg/kg) zinc hydroaspartate administration (*ip*) increases the extracellular zinc by 48% in the rat prefrontal cortex. These data for the first time demonstrate: 1) utility of ASV zinc detection in brain microdialysates and 2) that single *ip* zinc administration increases brain (cortical) extracellular zinc pool. The results indicate zinc-induced fast brain penetration and may explain its rapid pharmacological effects.

Key words: *zinc treatment, extracellular zinc, stripping voltammetry, microdialysis, prefrontal cortex, rat*

INTRODUCTION

Zinc is involved in hundreds of biochemical processes. The high zinc concentration is localized in the brain, particularly in the hippocampus and cerebral cortex (1). Zinc is bound to enzyme or other proteins, stored in presynaptic vesicles of glutamatergic (hippocampus, cortex) zinc containing

neurons or present extracellularly in free form (2). Zinc modulate central neurotransmission mostly *via* glutamate receptors, while other receptors and channels are also influenced by this ion (3, 4). Zinc exhibits high potency in inhibiting NMDA receptors, however, potentiation of the AMPA and inhibition of mGluR5 glutamate receptors is also powerful (3).

Disturbed zinc homeostasis was demonstrated in many neuro-psychiatric illnesses (1).

Diminished zinc blood (serum, plasma) concentration has been demonstrated in depressed patients and in most studies successful antidepressant treatment normalized this zinc level (5-10). Preclinical tests/models demonstrated antidepressant potential of zinc salts (11).

Previously, the increase in hippocampal synaptic zinc concentrations following repeated (but not acute) treatment with zinc hydroaspartate and electroconvulsive shocks were demonstrated (12, 13). Since cerebral cortex is also involved in the psychopathology and therapy of psychiatric disorders (14 - 17), in the present study, we investigated the effect of acute zinc administration on the extracellular zinc concentrations in the rat prefrontal cortex.

We used anodic stripping voltammetric (ASV) for zinc ions determination in brain microdialysates (which assays extracellular zinc). The dose/treatment schedule of zinc was previously examined and was effective in behavioral and biochemical experiments (18).

MATERIALS AND METHODS

Animals

The experiments were carried out on male Wistar rats (280 - 320 g). The animals were kept under 12:12 light-dark cycle with free access to food and water. The experimental group consisted of 7 animals. Zinc hydroaspartate (Farmapol, Poznan, Poland) at a dose of 65 mg/kg (11.5 mgZn/kg of zinc) was administered intraperitoneally. All procedures were conducted in compliance with National Institutes of Health Animal Care and Use Committee guidelines, and were approved by the Ethics Committee of the Institute of Pharmacology, Krakow.

In vivo microdialysis

One day before the experiment, the rats were anesthetized with ketamine (75 mg/kg *i.m.*) and xylazine (10 mg/kg *i.m.*) and placed into a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The scalp was retracted and holes drilled trough the skull for insertion of vertical microdialysis probe into prefrontal cortex (2.7 mm anterior from the bregma, 1.0 mm lateral from the sagittal suture and 4.5 mm ventral from the dural surface) (*Fig. 1*) (19). Microdialysis probes were constructed by inserting two fused silica tubes (30 and 35 mm long, 150 µm outer diameter (o.d.); Polymicro Technologies Inc., Phoenix, AZ) into a microdialysis fiber (220 µm o.d.; AN69, Hospal, Bologna, Italy). The tube assembly was placed in a stainless steel cannula (22 gauge, 10 mm) forming the shaft of the probe. Portions of the inlet and outlet tubes were individually placed inside

polyethylene PE-10 tubing and were glued for protection. The free end of the dialysis fiber was sealed, and 3 mm of the exposed length used for dialysis in the prefrontal cortex.

One day after the surgery and probe implantation, the inlet of the dialysis probes was connected to a syringe pump (BAS, IN, USA) which delivered an artificial cerebrospinal fluid (aCSF) composed of [in mM]: NaCl 145, KCl 2.7, $MgCl_2$ 1.0, $CaCl_2$ 1.2; pH = 7.4 at a flow 1.5 µl/min. After 3 h of washing period, when the extracellular level of zinc became stable, 2 baseline samples were collected every 40 min from freely moving animals. Next, zinc hydroaspartate was *ip* injected into animals and consequent dialysate fractions were collected every 40 min for 160 min.

At the end of the experiment, the rats were sacrificed and their brains were histologically examined to validate the correct probe placement (*Fig. 1*).

Zinc determination
Apparatus and software

A multipurpose Electrochemical Analyzer M161 with the electrode stand M164 (both MTM-ANKO, Poland) were used for all voltammetric measurements. The classical three-electrode quartz cell, volume 5 mL, consisting of the Control Growth Mercury Drop Electrode type M164 (MTM-ANKO, Poland) with the surface area of 1.4 mm^2 as a working electrode, used in Hanging Mercury Drop Electrode (*HMDE*) mode, a double junction reference electrode Ag/AgCl/3M KCl with replaceable outer junction (2.5 M KNO_3) and a platinum wire as an auxiliary electrode. Before the measurements, and to avoid accidental contamination, the voltammetric cell was rinsed at first with

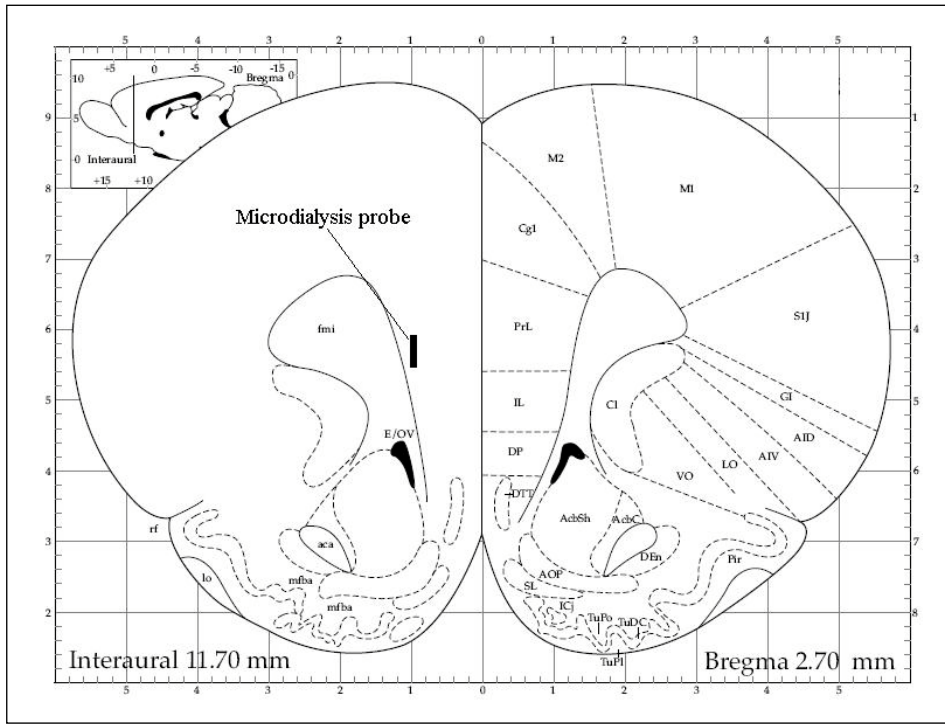

Fig. 1. Photomicrograph showing prefrontal cortical perfusion localization.

2 M HNO_3 (Suprapure) and then many times with distilled water. Magnetic Teflon-coated bar was used for stirring (approximately 400 rpm.) during the accumulation period. All experiments were carried out at room temperature, 20 ± 2 °C. Liquid samples were mineralized using a UV-digester (Mineral, Poland). The MTM-ANKO *EAGRAPH* software enabled electrochemical measurements, data acquisition and advanced processing of the results.

Reagents

All used reagents were of analytical grade. All solutions and the sample preparation were made using quadruple distilled water (two last stages from quartz). HNO_3 65%, H_2O_2 30% and KNO_3 (Merck, Suprapur®) were used for the preparation of samples. The standard solution of zinc(II) was at a concentration of 1000 $mg \cdot L^{-1}$ (Merck). Solutions with lower zinc concentrations were made weekly by appropriate dilution of the stock solution. Fumed silica of the specific surface area 255 $m^2\ g^{-1}$ was obtained from Sigma-Aldrich. Fumed silica was activated by roasting for 30 min in 950 °C. All glassware prior to analysis was soaked in 6 M nitric acid and rinsed several times with distilled water.

Sample preparation

Brain microdialysate samples were acidified with nitric acid immediately after collection by addition of 1 µL HNO_3 (conc.) to each 30 µL of sample. The pretreatment, intended to destroy organic compounds (complexants and organic surfactants), followed the wet ashing method used in the ASV determination of Zn^{2+} in microdialysates. Then, samples were transferred directly into a miniature quartz tube and were digested by UV irradiation for 2 h. The quartz tube were let to cool to room temperature. Aliquots of 20 µL of this solution were introduced into the electrochemical cell containing 2 mL of the supporting electrolyte.

ASV procedure

The stripping was performed in the differential pulse (DP) mode. 2 mL of 0.05 M KNO_3 was added in the electrochemical cell as a blank and the solution was purged with the argon of 99.995% purity for at least 5-7 min. The preconcentration step was carried out from the stirred solution for a period of t_{acc} = 20 s at a E_{acc} = -1.05 V versus Ag/AgCl, at a fresh mercury drop. After a rest period of 5 s, differential pulse voltammogram was recorded in the anodic direction from -1.05 to -0.8 V with a potential scan rate of 25 $mV \cdot s^{-1}$ and pulse amplitude of -50 mV. The voltammogram for the blank solution demonstrates electrochemical cell and supporting electrolyte purity. Then, twenty micro liters of sample solution was added to the cell while maintaining an argon atmosphere over the solution and the differential pulse voltammograms were recorded. The quantitative determinations of zinc ions were performed using the standard additions method (three concentrations). Three curves were recorded and averaged for each concentrations. All samples were measured at the same conditions.

When UV mineralization was not satisfactory (detected as high background) the fumed silica was added directly to the sample. SiO_2 is fast and efficient adsorbent which may be used for surfactant removal directly in voltammetric cell. Usage of fumed silica directly in voltammetric cell is restricted to amount of 5 mg per 1 mL.

Influence of DP ASV parameters on technique on zinc(II) peak

In order to adapt the differential pulse anodic stripping voltammetry (DP ASV) method to nanomolar concentrations of Zn^{2+} three parameters were optimized: pulse amplitude (ΔE), step

potential (E_s) and pulse time ($t_p = t_w$ (waiting time) + t_s (current sampling time)). Consequently, these parameters were investigated. To optimize the conditions for Zn^{2+} measurements, the following instrumental parameters were systematically verified: E_s in the range 1 - 5 mV; ΔE in the range 10-60 mV and t_p from 10 to 60 ms. Changes of the potential step (in the given range) cause the increase on peak current by ca. 10%. However, increasing E_s is accompany by the background current rising. Therefore, it is not suggested to apply the $E_s > 2$ mV. The step potential of 2 mV was applied in further work. For a pulse amplitude of 10 mV the lead peak current for supporting electrolyte containing 20 nM Zn^{2+} was equal to by ~3 nA and increased with increasing pulse amplitude. For ΔE, changing in the range 10-60 mV the width of the peak at half-height was 48-65 mV. The peak currents were similar for both negative and positive amplitude values. The pulse amplitude of -50 mV was chosen. The pulse width was changed from 10-60 ms. In each case, $t_w = t_s$ (waiting time = current sampling time). The high of the zinc peak decreases according to the increasing time t_p but also background current decreases. The best result (precision, reproducibility, and the relation between the peak and background current) was obtained for t_p = 40 ms, and this was the value chosen for further study.

Effect of accumulation time and potential

The influence of the accumulation potential was studied in the range from -1.15 to -1.0 V with supporting electrolyte spiked with 20 nM Zn^{2+}. The repeatability and the magnitude of the analytical signal were found to be independent of the accumulation potential in the potential range -1.15 to -1.05 V. The accumulation potential -1.05 V was chosen. The accumulation time was changed from 10 to 60 s. The peak current for supporting electrolyte containing 20 nM Zn^{2+} increased linearly with the accumulation times. For further study the accumulation time of 20 s was chosen. The zinc peak potential is not dependent on either the accumulation time or potential.

The calibration graph for Zn^{2+} for the accumulation time of 20 s was linear from 1 to 100 nM and obeyed the equation $y = 0.58 \pm 0.07x$ [nA/nM] + 0.35 ± 0.05 [nA]. The correlation coefficient was r = 0.9995. The relative standard deviation for Zn^{2+} determination at the concentration 20 nM was 2.2% (n = 5). The detection limit for Zn^{2+} following the accumulation time of 20 s calculated as a 3σ for the blank, was equal to 1 nM.

Data analysis

The statistical significance of the results was estimated by using one-way ANOVA and Tukey's post hoc test. All results are presented as mean ± S.E.M.; $p<0.05$ was considered as statistically significant.

RESULTS

Analysis of standard Zn^{2+} concentrations

The representative voltammetric curves are presented in *Fig. 2.* The shape and width of the peak were similar to that obtained from a synthetic solution. The obtained values of standard (10 nM), based on three replicates, was 10.4 ± 0.6 nM of Zn^{2+} ions. The detection limit for the determination of zinc under these conditions was estimated to be 1 nM. The recovery of Zn^{2+} was tested by addition of 10 nM of Zn^{2+} (using the standard additions method). The average recovery was 102 ± 3%.

Analysis of Zn^{2+} concentration in brain microdialysate samples

The basal concentration of zinc ion in microdialysate was 1.01 ± 0.11 μM. A 48% rise of this basal concentration 40 min following zinc hydroaspartate

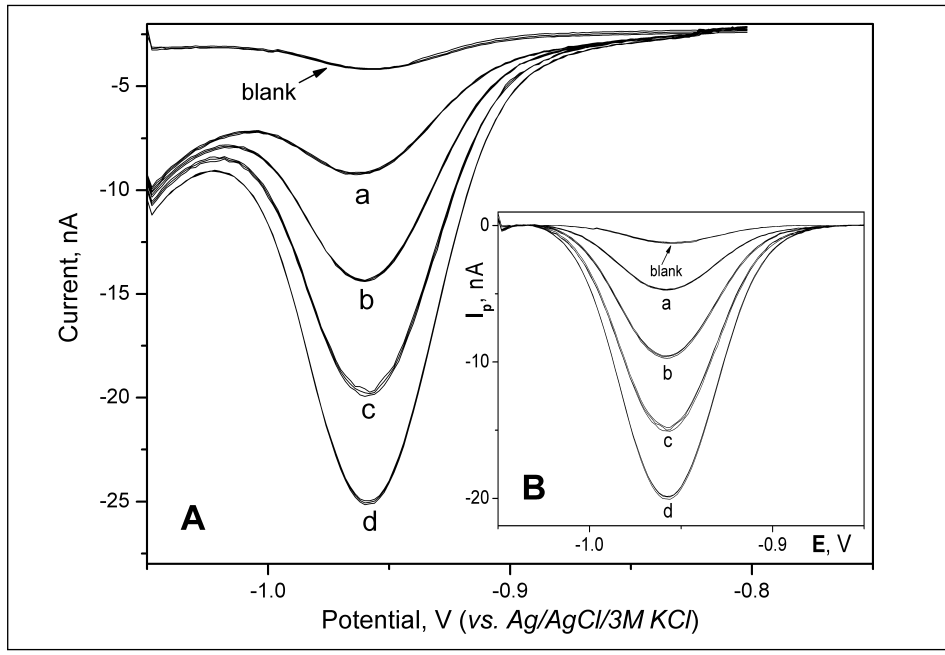

Fig. 2. (A) DP ASV voltammograms of Zn^{2+} ions in microdialysate sample on the hanging mercury drop electrode (CGMDE) in deaerated supporting electrolyte. *Curves*: blank 2 mL of 0.05 M KNO_3; (a) + 20 μL of microdialysate sample; (b, c and d) + 10, 20 and 30 nM Zn^{2+}, respectively. DP mode: pulse amplitude, -50 mV; pulse width (waiting time + current sampling time), 40 ms; potential step, 2 mV. Condition of electrodeposition: E_{acc} = -1.05 V; t_{acc} = 20 s. (B) curves from part (A) following background correction.

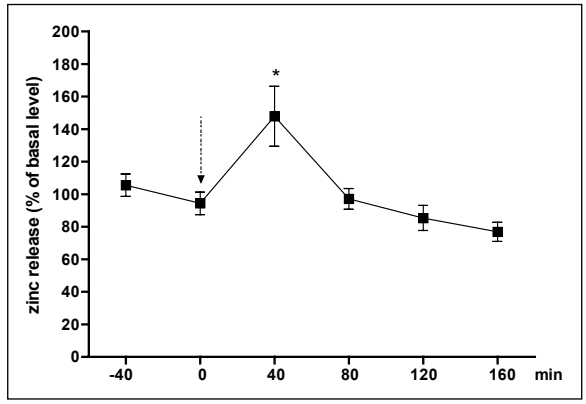

Fig. 3. Time course of the effect of zinc hydroaspartate (65 mg/kg, *i.p.*) administration (arrow) on extracellular concentration of zinc in the prefrontal cortex. Value are mean ± SEM, n = 7 rats. ANOVA: $F(4,25) = 10.66$, $P < 0.0001$. *P<0.01, *vs*. basal level (Tukey's post hoc test).

injection was demonstrated (*Fig. 3*). Then, this effect was gradually disappearing (*Fig. 3*).

DISCUSSION

Zinc exhibits antidepressant activity in preclinical tests/models of depression and also adjunctive activity to antidepressant therapy in human depression was reported. Thus, zinc is active in the forced swim and tail suspension tests (18, 20 - 22), olfactory bulbectomy (18), chronic unpredictable stress (23) and chronic mild stress (our unpublished data). Further, zinc enhance antidepressant-like activity of antidepressants in forced swim test and chronic unpredictable stress (23, 24), which suggests such effect in human depression. In fact our clinical studies demonstrated zinc activity as an adjunct treatment to antidepressant drugs (25). However, the mechanism of zinc antidepressant activity is not quite understood. The main zinc mechanism is related to the antagonism of the NMDA receptor complex, while antagonism to the group I mGlu receptors and enhancing of the AMPA receptors should also be considered. Moreover, the induction of BDNF may be also related to antidepressant zinc activity (11, 26, 27).

The main question is how pharmacokinetically/pharmacodynamically zinc execute the antidepressant action? Previously we demonstrated that chronic (but not acute) zinc treatment increases synaptic zinc concentration in rat hippocampus (12). Such an effect resembles effect of electroconvulsive treatment (ECT)(13, 28). However, antidepressant drugs do not influenced hippocampal synaptic zinc concentrations (28). Thus, as was suggested previously (12) the increase of the synaptic zinc may be connected with "specific" (*e.g.* shorter onset) antidepressant activity of both treatments. Fast antidepressant effect of ECT was demonstrated in human depression (29), whereas fast zinc activity may be predicted from preclinical studies (27). Indeed, zinc fast action was demonstrated in olfactory bulbectomy model, as zinc antidepressant-like effect appeared following acute administration while antidepressants need repeated administration to achieve such effect (18). Moreover, only one week of zinc treatment was sufficient to induce antidepressant-like action in chronic mild stress model, when *e.g.* activity of imipramine developed after 3 weeks of treatment (our unpublished data). Since, besides hippocampal also frontal cortical (14, 15) abnormalities were demonstrated in human depression, in the present study we examined frontal cortical zinc alterations induced by zinc administration to rats.

Voltammetric techniques are based on the recording of the current, *i*, which flows between the working electrode and an auxiliary electrode. Due to the reduction or oxidation of the test element, as function of the potential, E, imposed on the working electrode and expressed with respect to that of a reference electrode.

Since the beginning of direct current (dc) polarography and voltammetry development, zinc was determined by dropping mercury electrode (DME). The walve for its reduction to the metal is reversible in the majority of common electrolytes. Zinc is reduced reversibly to the metal at mercury electrodes, in neutral solutions the $E_{1/2}$ is about -1.0 V. On the other hand, in acid solutions, the zinc wave merges with that for H^+ reduction (30).

The sensitive determination of zinc traces is quite simple by ASV equipped with stationary hanging mercury drop electrode (HMDE). In ASV, the metal, Me, is preconcentrated in Hg, in a first step, by reduction of its ion, Me^{n+}, at constant potential and the reoxydation peak is measured in a second step. The determination of zinc ions according to the stripping process is the result of the overall two-electron oxidation process $Zn(Hg) \rightarrow Zn^{2+} + 2e$.

In the present study we employed the anodic stripping voltammetric method for zinc ions determination in fronto-cortical dialysates. This method was introduced by Omanovic et al (31) for detection of low concentrations of trace metals (Zn, Cd, Pb and Cu) in aquatic solutions. Then this method was applied in biological systems. We used this ASV method to determine zinc level in whole mouse brains (32), where zinc concentrations ranged between 5-12 μg/g of tissue. The other group (33) determined zinc in whole blood, where μM concentrations were detected.

During evaluation of the sensitivity of this voltammetric method we established that the nM concentrations of zinc was easily detectable, thus, we used this procedure to examine zinc concentrations in brain microdialysate. Presently, we demonstrate a 48% increase in extracellular zinc concentration in fronto-cortical region following 65 mg/kg of zinc hydroaspartate (11.5 mgZn/kg) *ip* administration. Thus, single administration of zinc increases extracellular zinc concentration in the brain, which indicate rapid zinc passing through the blood-brain barrier. The assay of zinc in brain microdialysate fluid was previously demonstrated using a flameless atomic absorption spectroscopy, where the extracellular zinc concentration in hippocampus or amygdala was 0.06 - 0.12 μM (34, 35). The concentration of zinc in our cortical measurement is 10x higher then in hippocampal or amygdala regions demonstrated in the above mentioned reports (34, 35), while is similar (1.8 μM) to hippocampal zinc extracellular concentration obtained by fluorescence method (36). However, the lowest extracellular zinc concentration (19 nM) was shown in hippocampus by a ligand-trapping technique (37). All the discrepancies, which may be due to differences in the brain structures, methods of zinc determinations and/or methodological approach, indicate the need for further detailed examinations of extracellular brain zinc concentration.

In summary, these data for the first time demonstrate:1) utility of ASV zinc detection in brain microdialysates and 2) that single *ip* zinc administration increases brain (cortical) extracellular (synaptic) zinc pool. The results

indicate zinc-induced fast brain penetration and may explain its rapid pharmacological effects.

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