# Effect of a Deficiency of Ceruloplasmin Copper in Blood Plasma on Copper Metabolism in the Brain

**P. S. Babich, N. V. Tsymbalenko, S. A. Klotchenko, N. A. Platonova, O. O. Masalova, E. A. Zatulovski, M. M. Shavlovskii, N. S. Sapronov, and L. V. Puchkova**

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Copper deficiency in adult rats was induced by addition of silver chloride to the feed. The concentrations of silver, copper, iron, and zinc and relative activity of genes for copper transporting proteins and copper enzymes were measured in the cortex, cerebellum, hippocampus, amygdala, pituitary gland, and hypothalamus. Silver was accumulated only in the hypothalamic—pituitary system. These changes were accompanied by a decrease in the concentration of copper and increase in the contents of iron and zinc. Activity of genes for copper transport enzymes (high-affinity copper transporter; and two copper transport ATPases, ATP7A and ATP7B) and copper enzymes that were formed in the intracellular secretory pathway did not decrease in the brain of rats with copper deficiency. Relative activity of genes for intracellular copper enzymes ($Cu^{2+}/Zn^{2+}$ superoxide dismutase and subunit IV of cytochrome *c* oxidase), concentration of immunoreactive polypeptides of superoxide dismutase, and enzymatic activity of superoxide dismutase remained unchanged under these conditions.

**Key Words:** *gene expression of copper transport proteins and copper enzymes; copper metabolism; Ag-induced copper deficiency*

Over the last decades, considerable amounts of silver ions were used for disinfection of drinking water. This treatment is followed by a multifold increase in silver content in the human organism. Silver does not belong to a group of trace elements. None of the physiological processes in any organism require the presence of Ag(I). However, the concentration of silver in living organisms of various phylogenetic groups is higher than in the environment. Therefore, the organism can receive and accumulate silver ions. The ion of Ag(I) is isoelectronic to the oxidized atom of copper (Cu(I)). Therefore, Ag(I) can use proteins for the safe transport of copper inside (to the site of copper enzyme formation) and outside the cell [5]. The proteins contain His-, Met-, and Cys-rich motifs coordinating the copper atom in an oxidized state of (Cu(I)), which is exchanged between these structures. Copper transport proteins bind and transfer Ag(I) [13] by the intracellular pathways that are similar to those for Cu(I). However, incorporation of Ag(I) into active sites of copper enzymes with other ligands can be blocked. When silver atom is incorporated into the active site, this atom cannot simulate functions of the copper atom. Due to oxidation-reduction activity, the copper atom provides function of the enzyme. Hence, an increase in silver consumption can modulate the formation of copper enzymes.

Here we studied the transport of copper and activity of copper enzymes in the brain of animals feeding a silver-enriched diet (Ag-rats).

Institute of Experimental Medicine, Russian Academy of Medical Sciences, St. Petersburg, Russia. ***Address for correspondence:*** puchkovalv@yandex.ru. L. V. Puchkova

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## MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing 180-200 g and obtained from the Rappolovo nursery. The animals were kept under standard conditions, fed a balanced diet, and had free access to water. AgCl in a daily dose of 50 mg/kg was added to the feed of treated animals. The blood was sampled from the cervical vessels. After blood clotting, the serum was isolated by centrifugation.

The study was performed with the cortex, cerebellum, hippocampus, pituitary gland, and hypothalamus. These structures differ in the copper status (profile and activity of expressed genes for copper transport proteins) [3]. We studied genes encoding the high-affinity copper importer (CTR1 protein) and two copper transport ATPases (ATP7A and ATP7B). Brain ATP7A is responsible for copper ion import and incorporation of these ions into copper enzymes of the intracellular secretory pathway. ATP7B plays a role in the release and incorporation of copper into secretory ceruloplasmin (CP) [5]. Activity of the CP gene was measured. Alternative splicing of the primary transcription product results in the formation of two mRNA. The first type of mRNA encodes the secretory form of CP (copper transport and iron import). And the second type of mRNA encodes CP, which is bound to the plasma membrane through the glycosyl phosphatidyl inositol anchor (GPI-CP, iron export) [6]. The CP gene is considered as a gene, which encodes the universal copper donor for non-hepatocyte cells (secretory CP) and two extracellular copper enzymes (CP and GPI-CP). We estimated relative activity of genes that encode two intracellular copper enzymes expressed in all cells ($Cu^{2+}$/$Zn^{2+}$ superoxide dismutase ($Cu^{2+}$/$Zn^{2+}$-SOD), SOD1) and isoform 1 of cytochrome *c* oxidase subunit IV (Cox4i1). The gene of subunit IV was selected as a gene encoding the subunit, which plays a crucial role in the assembly of the mature complex of cytochrome *c* oxidase. Since this gene is a nuclear intron gene, it can be used for the RT-PCR analysis [11]. We evaluated relative activity of a gene encoding the β-amyloid protein precursor (*APP*). *APP* probably plays a role in copper transport to the cell, which is associated with the reduction of Cu(II) into Cu(I) near the cell surface [10]. The studied genes encode the proteins that determine the pathways of copper transport from the extracellular space to the site of incorporation into secretory and intracellular copper enzymes.

The preparation and purity evaluation of total RNA, sequences of PCR primers, conditions of the RT-PCR analysis, procedure of immunoblotting, isolation of the cytosol, and measurement of SOD1 activity were described previously [1,3]. Experiments were performed with rabbit antibodies to glyceraldehyde-3-phosphate dehydrogenase and full-size recombinant SOD1 (Abcam) and horseradish peroxidase-conjugated antibodies to rabbit γ-immunoglobulins (Amersham). Oxidase activity of CP was measured by the method of Ravin with *p*-phenylenediamine. Metal concentration was measured by atomic absorption spectrometry with electrothermal atomization and Zeeman correction of nonselective absorption on a Perkin-Elmer spectrometer (Model 4100ZL) [3]. The results were analyzed by Student's *t* test. The differences were significant at $p<0.05$.

## RESULTS

The development of copper deficiency in Ag-rats was confirmed by the disappearance of azide ion-sensitive phenoloxidase activity from blood serum (oxidase activity of CP) [2]. Oxidase activity was detected in serum samples from the caudal artery of Ag-rats on weeks 2, 3, and 4 of feeding. Oxidase activity decreased gradually up to the 3rd week and was not detected after 4 weeks (in most animals). Therefore, the experiment was performed on animals feeding AgCl for 4 weeks and having a blood copper concentration of 100 μg/liter (*vs*. 1130 μg/liter in the control). Not more than 50% of copper ions (total concentration 100 μg/liter) could be bound to CP. The relative concentration of immunoreactive CP (estimated by semiquantitative immunoblotting) remained unchanged under these conditions. However, CP was presented by the apo-form [2]. Further studies were conducted on rats that had a copper deficiency in the blood. Copper was not supplied to brain cells of these animals for at least 1 week.

The main portion of silver in Ag-rats was found in the liver. Small amounts of silver were detected in the brain (Table 1). A detailed study showed that silver is regularly distributed in the brain of control animals. Silver concentration in the brain of these specimens was 1.2 μg/g wet tissue (Fig. 1). This level is slightly lower than the mean concentration of silver in mammals. In Ag-rats silver was accumulated only in the pituitary gland, where its concentration 2-fold surpassed the baseline (Fig. 1). The concentrations of iron and

**TABLE 1.** Silver Distribution in Ag-rats

| Organ | *n* | Silver concentration, μg/g wet tissue |
|---|---|---|
| Liver | 5 | 85.1±7.3 |
| Kidneys | 5 | 9.5±0.6 |
| Brain | 5 | 2.3±0.4 |
| Testes | 2 | 1.5 |

zinc were significantly elevated in the pituitary gland (by 5 and 2 times, respectively). Copper concentration was reduced only in the hypothalamus (Fig. 1). We conclude that changes in the content of copper, iron, and zinc are observed only in the hypothalamic—pituitary system of Ag-rats. Our findings confirm the important role of copper metabolism in this structure of the brain. Phenoloxidase activity is detected in the content of the third ventricle. Estradiol treatment is accompanied by the increase in phenoloxidase activity. Blood CP cannot cross the blood-brain barrier. Hence, secretory CP is synthesized in the hypothalamic—pituitary system. These data are consistent with the results of our previous experiments (*in vivo*). We showed that CP gene expression and copper concentration in the brain are highest in the hypothalamic—pituitary system [3]. These features are probably related to a considerable volume and high rate of synthesis of brain-specific copper enzymes peptidylglycine α-amidating monooxygenase and dopamine β-hydroxylase, which play a role in the formation of neuropeptides and synthesis of neurotransmitters. These data explain the fact that an inhibitory effect of silver on copper metabolism is particularly pronounced in the hypothalamus and pituitary gland. The inability of copper transport proteins to distinguish between the copper ion and silver ion is consistent with the results of *ab initio* calculation with the quantum-chemical software GAMESS. The binding energy of Cu(I) and Ag(I) with the cytosolic copper-binding motif of the CTR1 protein (His-Cys-His) and motif of Cu-chaperons (Cys-X-X-Cys), as well as the transfer energy from CTR1 to Cu-chaperons, practically did not differ between both ions (r~0.91).

RT-PCR did not reveal the relative concentration of mRNA for CP, GPI-CP, ATP7A, and ATP7B in various brain structures of Ag-rats, including the vascular plexus (with cells of the endothelial type; Fig. 2). mRNA for GPI-CP and ATP7A was mainly expressed in brain structures of control rats. These results are consistent with published data [3,4,12]. The activity of *CTR1* and *APP* genes was significantly reduced in Ag-rats (Fig. 2, *e*, *f*). Therefore, the activity of genes for copper transport proteins decreases in the absence of copper donors in brain cells. Our results serve as indirect evidence for the existence of a regulatory mechanism for these genes. This mecha-

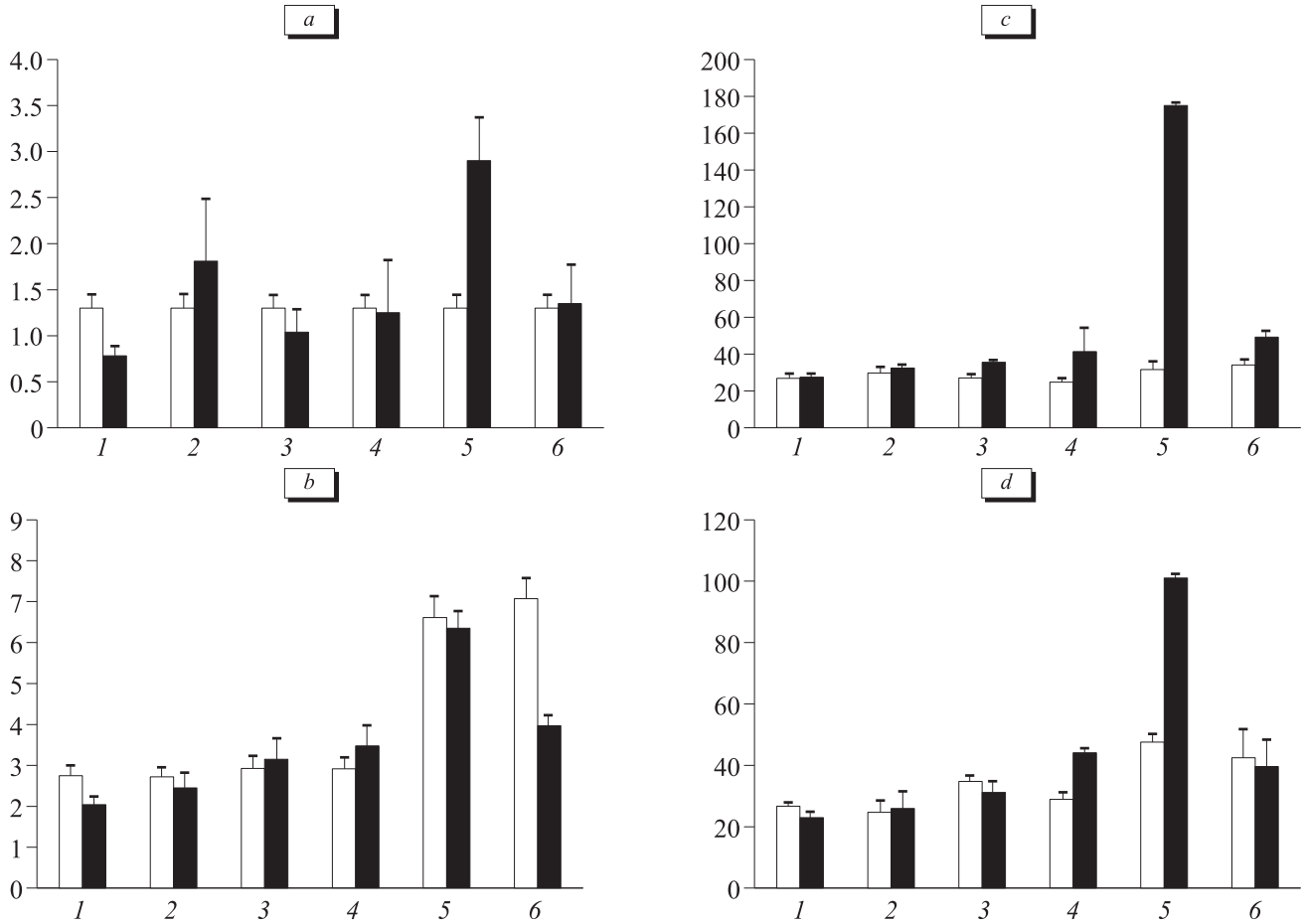


**Fig. 1.** Concentration of silver (*a*), copper (*b*), iron (*c*), and zinc (*d*) in rat brain regions. Cortex (*1*), cerebellum (*2*), hippocampus (*3*), amygdala (*4*), pituitary gland (*5*), and hypothalamus (*6*). Ordinate: μg/g wet tissue. Light bars, control; dark bars, Ag-rats.

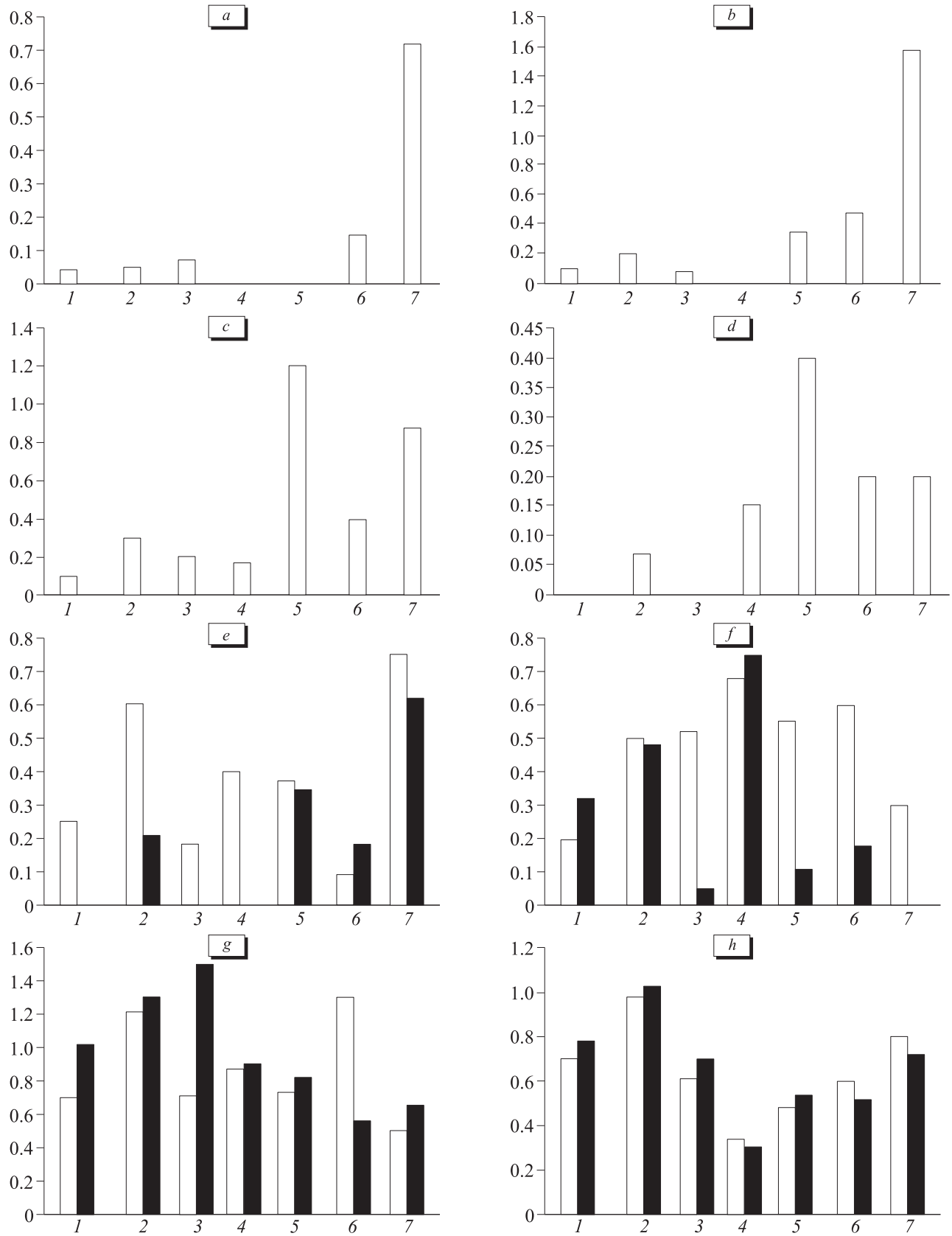


**Fig. 2.** Relative activity of genes for copper transport proteins and copper enzymes in brain regions of Ag-rats (RT-PCR analysis). mRNA for secretory CP (*a*); mRNA for GPI-CP (*b*); ATP7A mRNA (*c*); ATP7B mRNA (*d*); CTR1 mRNA (*e*); APP mRNA (*f*); SOD1 mRNA (*g*); Cox4i1 mRNA (*h*). Cortex (*1*), cerebellum (*2*), hippocampus (*3*), amygdala (*4*), pituitary gland (*5*), hypothalamus (*6*), and vascular plexus (*7*).

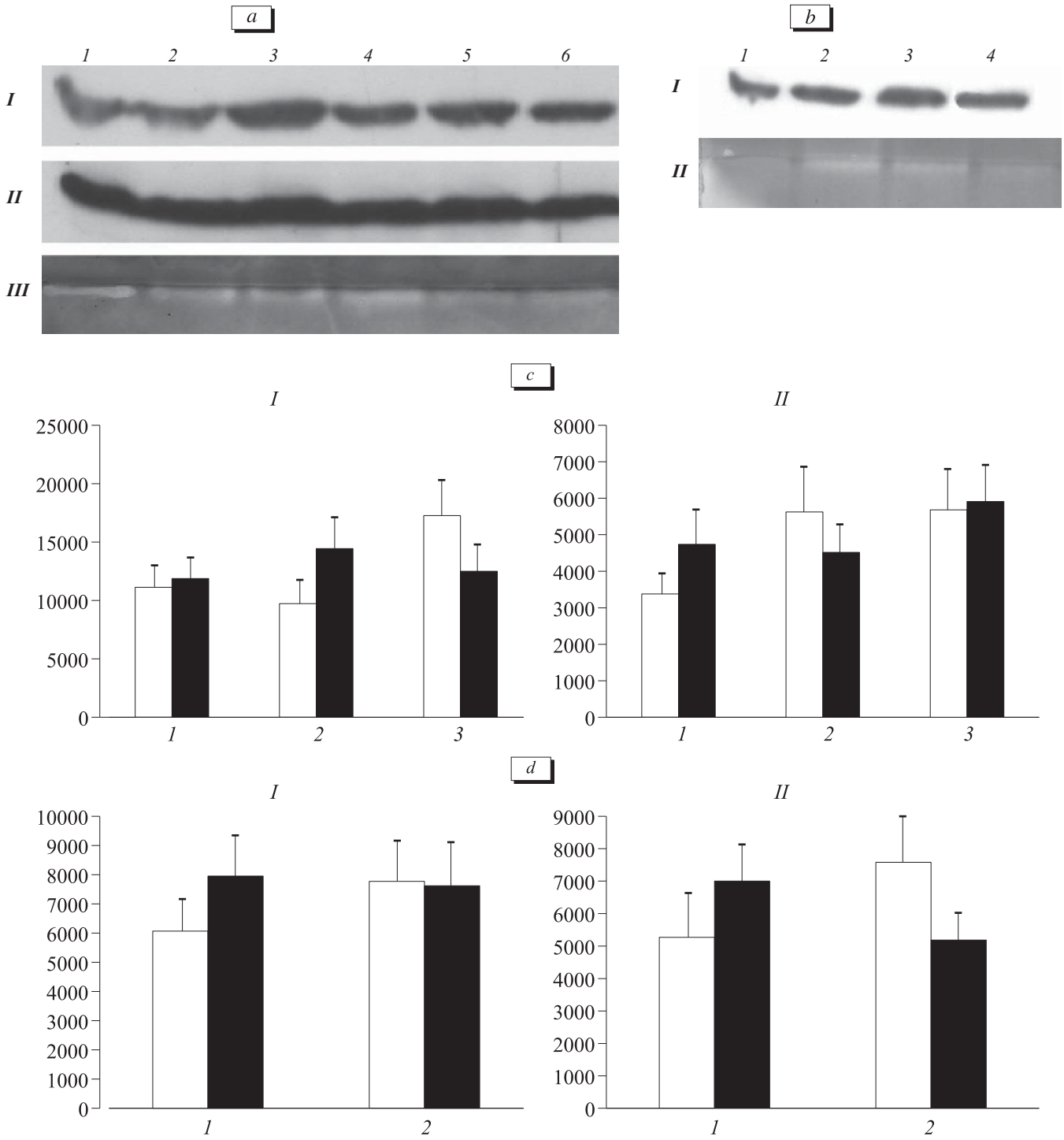


**Fig. 3.** Intracellular distribution and activity of SOD1 in brain regions of Ag-rats. (*a*) Immunoblotting of the cytosol from the cortex (*1*), cerebellum (*2*), and hippocampus (*3*) of control animals and Ag-rats (*4*, *5*, and *6*, respectively). (*I*) Antibodies to SOD1. *II*: Antibodies to glyceraldehyde-3-phosphate dehydrogenase. Electrophoresis in 10% PAAG in the presence of sodium dodecyl sulfate. *III*: The same. The gel was stained for SOD1 activity after electrophoresis in 10% PAAG under non-denaturing conditions. *b*) Immunoblotting of MIMS from the cortex (*1*, *3*) and cerebellum (*2*, *4*) of controls (*1*, *2*) and Ag-rats (*3*, *4*). *I*: Immunoblotting with antibodies to SOD1 after electrophoresis in 10% PAAG in the presence of sodium dodecyl sulfate. *II*: SOD1 activity in the gel after electrophoresis under non-denaturing conditions. *c*) Relative content (*I*) and activity of SOD1 (*II*) in the cytosol from the cortex (*1*), cerebellum (*2*), and hippocampus (*3*). Light bars, control; dark bars, Ag-rats. *d*) Relative content (*I*) and activity of SOD1 (*II*) in MIMS from the cortex (*1*) and cerebellum (*2*). Light bars, control; dark bars, Ag-rats. Ordinate, density of zones, rel. units. The content of all samples was equalized by protein concentration (30 μg total protein per sample, estimated by the Bradford method). The regularity of protein transfer to nitrocellulose filters was controlled by staining of filters with Ponceau solution. The filters were scanned after staining. Various filters were compared for protein content in zones. The data are presented as mean values of 3 measurements.

nism probably mediates the modulation of activity of unidentified transcription factor(s) by copper ions. Iron accumulation in the pituitary gland is probably related to changes in copper metabolism (*e.g.*, inactivation of the CP gene whose product (GPI-CP) plays a role in iron export from the cell) [8].

However, the expression of genes for intracellular copper enzymes SOD1 and Cox4i1 remained practically unchanged in brain structures (Fig. 2, *g*, *h*). The exception was the hypothalamus. The concentration of mRNA for SOD1 was reduced by 2 times in the hypothalamus. The content of immunoreactive polypeptides of SOD1 in the cytosol from cortical, cerebellar, and hippocampal cells (immunoblotting analysis; Fig. 3, *a*, *c*), as well as activity of SOD1, did not change in Ag-rats. These data are consistent with the results of RT-PCR for brain regions.

SOD1 is present not only in the cytosol, but also in the mitochondrial intermembrane space (MIMS) of mammalian and yeast cells [9]. Mitochondrial Mn-dependent SOD2 is absent in MIMS. SOD1 neutralizes superoxide radicals, protects cardiolipin from oxidation, and prevents mitochondrion-dependent apoptosis [7]. During the impairment of copper transport caused by defects in genes copper transport proteins, apo-SOD1 and copper chaperon (apo-form) are redistributed from the cytosol to MIMS. Apo-SOD1 is converted into the holo-form [9]. An immunoblotting analysis showed that the content of immunoreactive polypeptides of SOD1 in the cerebellum is similar in control animals and Ag-rats (Fig. 3, *b*, *d*). No differences were found in SOD2 gene expression in brain structures of Ag-rats and control specimens. The localization of SOD1 in cortical MIMS is not associated with antioxidant activity of this enzyme. Under conditions of copper deficiency, the mitochondrial pool of copper probably serves as a source of this substance [7].

Our results indicate that silver ions suppress the incorporation of copper into CP, which is synthesized in the liver. Silver ions have an inhibitory effect on copper metabolism in the brain. It is manifested in a sharp decrease in gene activity for the copper transport system (*e.g.*, gene for the extracellular copper transporter CP) and impairment of iron export form neurons. Further studies should be performed to provide scientific recommendations for the use of silver in disinfection.

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