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Research paper

Primary mechanism responsible for age-dependent neuronal dehydration

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Abstract

Neuronal dehydration and high $[Ca^{2+}]_i$ are essential hallmarks for age-dependent memory impairment. Na^+/K^+-ATPase, having membrane transporting and intracellular signaling functions, has age-induced dysfunctional character. Therefore, it could have a key role in age-dependent neuronal dehydration and increase of $[Ca^{2+}]_i$. However, it is not clear the dysfunction of which above mentioned functions of Na^+/K^+-ATPase serves as a primary mechanism for generation of age-dependent neuronal dehydration and increase of $[Ca^{2+}]_i$. In present work age-dependent effects of $\leq 10^{-9}$M (agonist for α_3 isoform which has only signaling function) and 10^{-4}M ouabain (agonist for α_1 isoform which has ion-transporting function) on brain cortex tissue hydration, $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ efflux through plasma membrane were studied. It was shown that $\leq 10^{-9}$M and 10^{-4}M ouabain concentrations have stimulation effects on cortex tissue hydration and Na^+/Ca^{2+} exchange in reverse mode. However, these effects have age-dependent weakening and increasing characters, respectively. It is suggested that $\leq 10^{-9}$M ouabain-induced tissue hydration is due to cAMP-activated Ca^{2+}-ATPase in endoplasmic reticulum membrane leading to reversion of Na^+/Ca^{2+} exchange and elevation of endogenous H_2O release in cytoplasm. This effect has age-dependent depressing character. Thus, the dysfunction of α_3 isoform-dependent intracellular signaling system could be considered as a primary mechanism for age-dependent neuronal dehydration.

Keywords: *Brain Cortex; $^{45}Ca^{2+}$ Efflux; $^{45}Ca^{2+}$ Uptake; Na^+/K^+-ATPase A_3 Isoform; Tissue Hydration.*

1. Introduction

Age-induced memory impairment during normal and pathological aging is one of the modern problems in neuroscience and has become a focal point for public health concern due to the aging of the world population.

The dysfunction of Na^+/K^+-pump, increase of $[Ca^{2+}]_i$, mitochondrial dysfunction and oxidative stress, increase of mitochondrial DNA and its damage, peroxidation of lipids, magnesium deficit, iron increase, formation of amyloid-β (Aβ) peptide and a number of other pathological changes in neurons described in literature are typical hallmarks for age-induced memory impairment which are accompanied by tissue dehydration [1]. However, which of the above mentioned mechanisms is primary in generation of age-induced memory impairment and cell death cascade is not clear yet.

It is known that neuronal death in brain, like in other cells, occurs by two phases; neuronal swelling (hydration) [2], [3] which is followed by its shrinkage (dehydration) [4], [5].

Earlier we have shown that neuronal swelling in hypo-osmotic medium has a discrete character [6] which leads to the increase of the number of functionally active proteins in membrane, having receptors [7], enzymes [8] and ionic channels forming properties [9]. At present, it is established that in cell membrane there are caveolae, containing different enzymes which are activated as a result of cell swelling [10]. Cell hydration-induced intracellular macromolecules' activity by "folding-unfolding" mechanism is another powerful pathway for regulation of intracellular metabolic activity [4], [11]. On the basis of the previous data that neuronal swelling in response of activation of ligand- and potential-activated ionic channels in neuronal membrane led to the increase of the number of Na^+/K^+-pump units ($[^3H]$-ouabain binding sides) in membrane [8], neuronal swelling was suggested as a protective reaction of cell [12].

At present it is well established, that cell swelling triggers proliferation, while its shrinkage promotes cell apoptosis [13], [14]. In spite of multi-regulatory role of cell hydration in regulation of cell functional activity, the role of neuronal dehydration in age-dependent memory impairment is contradictory and inconsistent [15].

The emerging evidence suggests that cell membrane is highly permeable for water, which is a universal messenger for signal transduction from bathing medium into intracellular metabolism. Because of the existence of special water channels (aqua pores) in membrane, water transport through membrane takes place much faster than predicted by simple osmotic diffusion [16]. Therefore, the osmotic gradient on membrane generated by extracellular or intracellular factors has a transient character and the factor-induced changes of intracellular water content are compensated by cell metabolism [17, 18, 19, 20], the dysfunction of which leads to cell pathology.

Na^+/K^+-pump, the dysfunction of which is a consequence of aging, has a key role in metabolic regulation of cell hydration [13], [10], [21], [22]. The latter is dictated by the facts that Na^+/K^+-pump has electrogenic character [23], [24] and generates Na^+ gradient on membrane which serves as an energy source for a number of sec-

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ondary ionic transporters in membrane, such as Na^+/H^+, Na^+/Ca^{2+}, Na^+/sugars, amino acids and osmolytes [17], [18], [21], [22], [25]. On the other hand Na^+/K^+-pump, being the highest adenosine triphosphate (ATP) energy utilizing mechanism, determines the rate of metabolic cascades of ATP synthesis. The latter is accompanied by intensive release of endogenous H_2O molecules in cytoplasm as a result of glucose oxidation [26]. Therefore, Na^+/K^+-pump could be considered not only as an ion-transporting mechanism in cell membrane as it was suggested by classical membrane theory [27] but also as a powerful mechanism regulating cell hydration through intracellular signaling systems. However, it is not clear the depression of which function (membrane ion-transporting or cytoplasm signaling system) of pump is a primary mechanism for age-induced neuronal dehydration.

Our previous study has shown that among three ouabain receptors (Na^+/K^+-ATPase), having low (α_1), middle (α_2) and high (α_3) affinities, α_3 isoform-dependent signaling system controlling cell hydration serves as an extra-sensitive and universal sensor for different chemical and physical signals [28], [29] and has pronounced age-dependent character [30], [31]. Therefore, we hypothesize that the dysfunction of Na^+/K^+-ATPase α_3 isoform-dependent signaling system could serve as a primary mechanism for generation of age-dependent neuronal dehydration [28]. Thus, the aim of the present study is to check this hypothesis, which stems from and is a natural extension of our previously published findings. We believe that the elucidation of this mechanism will serve as a novel therapeutic target for counteracting age-dependent neuronal dehydration.

2. Materials and methods

2.1. Animals

All procedures performed on animals were carried out following the protocols approved by Animal Care and Use Committee of Life Sciences International Postgraduate Educational Centre (LSIPEC, Yerevan, Armenia).

Experiments were performed on 405 young (six weeks old) and 405 old (18 months old) male albino rats. They were regularly examined, kept under control of the veterinary in LSIPEC and reserved in a specific pathogen-free animal room under optimum conditions of 12 h light/dark cycle, at temperature of 22 ± 2°C, a relative humidity of 50% and were fed ad libitum on a standard lab chow and water.

2.2. Chemicals

Tyrode's physiological solution (PS) containing (in mM) 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.05 $MgCl_2$, 5 $C_6H_{12}O_6$, 11.9 $NaHCO_3$, 0.42 NaH_2PO_4 and adjusted to pH 7.4 with NaOH was used. In order to receive 50% PS, 68.5mM of NaCl was replaced with 2M of non-metabolizing substance mannitol dissolved in Tyrode's PS for maintaining the osmolarity of solution. All chemicals were obtained from "Medisar" Industrial Chemical Importation Company (Yerevan, Armenia). K^+-free solution consisted of 5.4mM NaCI instead of 5.4mM KCI. $^{45}Ca^{2+}$ with specific activity 40mCi/ml (PerkinElmer, Massachusetts, USA) was added in K^+-free solution containing 68.5mM (50%) NaCl and used for the enrichment of tissue. [^{3}H]-ouabain with specific activity (25.34 Ci/mM) and non-radioactive ouabain (PerkinElmer, Massachusetts, USA) from 10^{-11}M to 10^{-4}M concentrations dissolved in physiological solution were used for intraperitoneal injections and tissues' incubation. $^{45}Ca^{2+}$ was used for intraperitoneal injections and tissues' incubation. The volume of injected solutions was adjusted according to the weight of animals (0.02ml/g), samples were incubated in 10 ml of experimental solution.

2.3. Tissue preparation

It is well known that anesthetics with different chemical and pharmacological profiles [32], [33], [34] significantly affect metabolic processes which play an important role in regulation of cell volume [13], [35]. Therefore, in present experiments animals were sharply immobilized by freezing method (dipping their noses into liquid nitrogen for 3-4 sec) and decapitated [36]. After such procedure the full absence of somatic reflexes on extra stimuli was recorded.

Based on our previous data [30] where the age-dependent kinetics of ouabain binding with α_3 receptors in brain cortex tissue is more pronounced than in subcortex and cerebellum tissues (in adult animals) the investigation of brain cortex tissue has been the subject of the present study.

In vivo experiments, 30 min before decapitation animals were intraperitoneally injected with investigated solutions. After this they were immobilized and decapitated. Investigated brain cortex tissue was isolated and dissected into samples weighing from 50 to 70 mg. In vitro experiments animals were firstly immobilized and decapitated. Their brain cortex tissue samples were dissected in the same manner and then incubated in investigated solutions. For each experimental group five animals were chosen. 8 samples were dissected from brain cortex of each animal. Thus, the average data for 3 independent experiments was calculated based on the data obtained from the study of 120 brain slices (40x3).

2.4. Definition of water content of brain tissues

Water content of brain cortex tissue was determined by traditional "tissue drying" method [37]. After measuring the cortex tissue wet weight (w.w.), tissue was dried in oven (Factory of Medical Equipment, Odessa, Ukraine) for 24 h at 105°C for determination of dry weight (d.w.). The quantity of water in 1g of d.w. tissue was counted by the following equation: (w.w. – d.w.) / d.w. [37].

2.5. Counting [^{3}H] ouabain receptors in membrane

To estimate the number of Na^+/K^+-ATPase molecules in membrane, the number of [^{3}H]-ouabain molecules binding with cell membrane was counted. In vivo experiments [^{3}H]-ouabain was intraperitioneally injected to animals. After 30 min animals were sacrificed and brain slices were dissected and incubated in 100 ml PS containing 1 mM "cold" ouabain. To remove surface-adherent and extracellular tracer the brain slices were washed fivefold, each wash about 5 min in duration, in PS containing 1 mM "cold" ouabain. After determination of wet and dry weights of samples, they were homogenized in 50 µl of 68% HNO_3 solution. Then 2 ml of Bray's scintillation fluid was added and chemoluminescence of samples was quantified with 1450-MicroBeta liquid scintillation counter (Wallac, Turku, Finland). The number of ouabain molecules binding with membranes of cells was defined per mg of dry weight of samples. The ouabain concentration injected to the animal was adjusted to animal body weight (10^{-x}M/g of animal weight, where 10^{-x} is the testing concentration of ouabain). The volume of the injected PS was also adjusted to the weight of animals (0.2 ml/g of animal weight). The lowest concentration of ouabain which was used in the experiments was 10^{-11}M [^{3}H] ouabain (isotope). Higher concentrations of ouabain used in the experiments contained 10^{-11}M [^{3}H] ouabain + 10^{-x}M cold (non-isotope) ouabain. Respective concentrations of ouabain used in vivo experiments were added in PS where brain slices were bathing.

2.6 Measurement of $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ efflux rate constant

$^{45}Ca^{2+}$ uptake by brain cortex tissue was measured by the following way: 0.0115mM $CaCl_2$ from 1.8 mM was substituted by radioactive $^{45}Ca^{2+}$ (11.2 mCi/l) in PS. In vivo experiments animals were intraperitioneally injected with $^{45}Ca^{2+}$ (with 0.187 mCi/g radioactivity of body weight). After 30 min animals were decapi-

tated and brain samples were incubated in ouabain-free PS (as a control) and PS containing 10^{-9}M and 10^{-4}M ouabain for 30 min. Then all samples were dried in oven during 24 h at 105°C. In vitro experiments brain tissue samples were pre-incubated in 10 ml cold (7°C) PS containing1.8 µl $^{45}Ca^{2+}$ (as a control) for 30 min and transferred into PS containing different concentrations of ouabain. Then they were dried in oven at 105°C for 24 h. The quantity of $^{45}Ca^{2+}$ uptake by brain slices was expressed by cpm/mg d. w.

For measurement of $^{45}Ca^{2+}$ efflux 240 brain slices for each young and old rats were pre-incubated for 60 min in K^+-free (containing 50% NaCl) cold (7°C) PS where $^{45}Ca^{2+}$ was added (1.8 µl $^{45}Ca^{2+}$ for 10ml PS) in order to enrich tissues by $^{45}Ca^{2+}$. Then $^{45}Ca^{2+}$-enriched samples were washed three times (in K^+-free solution) for 10, 5 and 5 min for removing extracellular $^{45}Ca^{2+}$. After the washout of 40 brain slices of young and old rats were placed in oven for 24 h at 105°C for detecting initial contents of $^{45}Ca^{2+}$. The rest 200 brain slices of young and old rats were divided into 5 groups, (40 slices in each group) and were incubated for 30 min in ouabain-free PS and 10^{-11}, 10^{-10}, 10^{-9} and 10^{-4}M ouabain containing PS. After this procedure they were dried in oven at 105°C for 24 h.

$^{45}Ca^{2+}$ content was measured by the same way as [^{3}H]-ouabain receptors as is described above. Taking the number of counts per minute (cpm) remaining in the samples at the end of experiments and adding back the cpm in each sample, the total radioactivity in tissue samples at the beginning of each collecting period was obtained. Then the rate constant of $^{45}Ca^{2+}$ efflux was calculated.

2.7 Statistic analysis

Microsoft Excel and Sigma-Plot (Version 8.02A, NY, and USA) were used for data analysis. Significance in comparison with the control group was calculated with Student's paired t - test with the following symbols (*P < 0.05; **P < 0.01; ***P < 0.001).

3. Results

3.1. Ouabain effect on brain cortex tissue hydration

As the major part (80%) of brain tissue consists of intracellular water [38], in present work tissue hydration serves as a parameter for estimation of intracellular water content in brain tissue.

It is known that inhibition of ion-transporting function of electrogenic Na^+/K^+-pump leads to neuronal hydration [17], [18], [19]. As the dysfunction of Na^+/K^+-pump is a consequence of aging, it is predicted that pump inhibition could cause more pronounced brain cortex tissue hydration in young animals than in old ones. However, the data presented in Fig. 1A indicate the opposite effect. Intraperitoneal injection with 10^{-4}M ouabain led to the increase of brain cortex tissue hydration which was more pronounced in brain tissues of old (16%) rats than those of young (7%) ones.

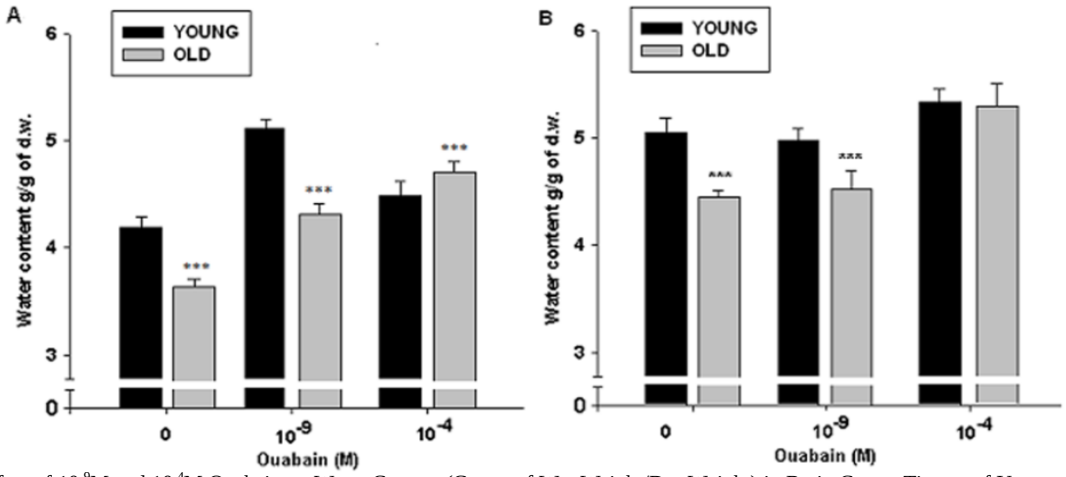


Fig. 1: The Effect of 10^{-9}M and 10^{-4}M Ouabain on Water Content (Grams of Wet Weight/Dry Weight) in Brain Cortex Tissues of Young and Old Rats in Vivo (A) and in Vitro (B) Experiments. Water Content of Brain Slices in Vivo Experiments was Determined after 30 Min of Intraperitoneal Injection Of Ouabain-Free and Ouabain-Containing (10^{-9}M and 10^{-4}M) PS. Water Content of Brain Slices in Vitro Experiments was Determined after 30 Min of Incubation of Brain Cortex Slices in Ouabain-Free and Ouabain-Containing (10^{-9}M and 10^{-4}M) PS. Black and White Bars Indicate the Water Content of Brain Cortex Tissues of Young and Old Rats, Respectively. Under Each Pair of Columns Corresponding Ouabain Concentrations are noted. Data of Each Pair of Bars are compared with Each other. Error Bars Indicate the Standard Error of the Mean (± SEM) of 120 Samples (40 X 3) for Three Independent Experiments. The Symbol (***) Indicates P<0.001.

In order to elucidate the role of initial metabolic state of brain tissue in determination of age-dependent increasing effect of 10^{-4}M ouabain on tissue hydration, the same protocol of experiments was also performed in vitro conditions where brain tissue samples were incubated in cold (7°C) PS. As can be seen in Fig. 1B, the depression of metabolic activity of tissue incubated in cold PS led to the increase of tissue hydration in both young (20%) and old (21%) animals compared with those of in vivo experiments (Fig. 1A). However, 10^{-4}M ouabain-produced additional tissue hydration lost its age-dependent character (Fig. 1B).

By our early work we have shown that in snail neurons >10^{-7}M ouabain concentrations have inactivation effect on Na^+/K^+-pump, while <10^{-7}M ouabain has activation effect on Na^+ efflux [8]. As can be seen in Fig. 1, in young animals intraperitoneal injection of 10^{-9}M ouabain (agonist for α_3 isoform) led to more pronounced increase of brain cortex tissue hydration (23.5%) than 10^{-4}M (13.5%) ouabain-induced inactivation of Na^+/K^+-pump. Moreover, the differences between the effects of 10^{-4}M and 10^{-9}M ouabain on tissue hydration had age-dependent decreasing character.

The facts that the increase of $[Ca^{2+}]_i$ serves as one of the essential hallmarks for aging [39] and $[Ca^{2+}]_i$ has a central role in intracellular signaling system of neurons [40], [41] allow us to suggest the increase of $[Ca^{2+}]_i$ as a potential candidate through which the intracellular signaling systems controlling age-dependent neuronal hydration can be realized. Therefore, to check this suggestion in next series of experiments age-dependent effects of different doses of ouabain on $^{45}Ca^{2+}$ uptake and efflux in brain tissue were studied.

3.2. Ouabain effect on $^{45}Ca^{2+}$ uptake

In Fig. 2A, data of experiments on $^{45}Ca^{2+}$uptake by tissue of brain cortex of young and old rats after intraperitoneal injections of $^{45}Ca^{2+}$-containing PS (control), PS containing ($^{45}Ca^{2+}$)+10^{-9}M and PS containing ($^{45}Ca^{2+}$)+10^{-4}M ouabain are presented. In Fig. 2B, data of experiments on $^{45}Ca^{2+}$ uptake by brain slices incubated in cold (7°C) PS containing $^{45}Ca^{2+}$, PS containing ($^{45}Ca^{2+}$)+10^{-9} and PS containing ($^{45}Ca^{2+}$)+10^{-4}M ouabain mediums are presented.

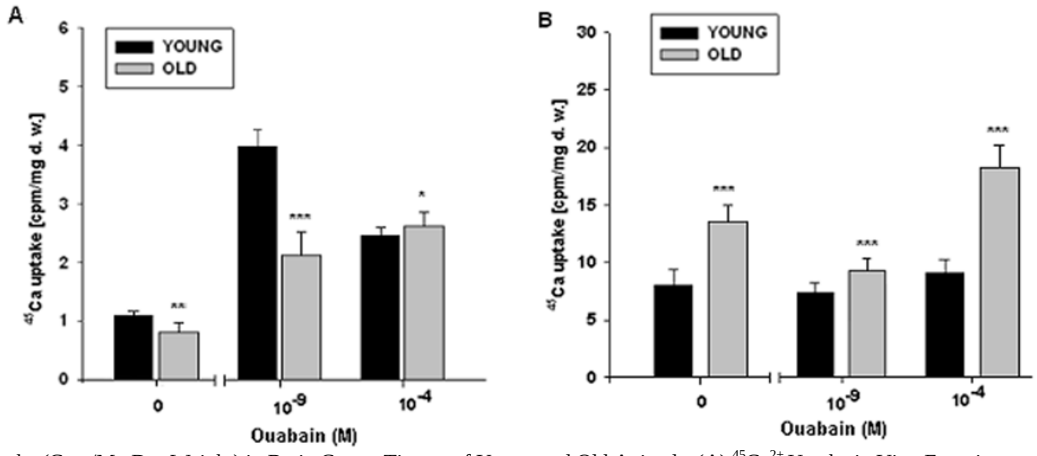


Fig. 2: $^{45}Ca^{2+}$ Uptake (Cpm/Mg Dry Weight) in Brain Cortex Tissues of Young and Old Animals. (A) $^{45}Ca^{2+}$ Uptake in Vivo Experiments Was Determined After 30 Min of Intraperitoneal Injection of Ouabain-Free and Ouabain-Containing (10^{-9}M and 10^{-4}M) PS. (B) Determination of $^{45}Ca^{2+}$ Uptake in Vitro Experiments Was Performed in the Same Conditions. The Size of Columns Indicates $^{45}Ca^{2+}$ Uptake by Tissue Expressed in Cpm/Mg Dry Weight. Under Each Pair of Columns Corresponding Ouabain Concentrations are noted. Error Bars Indicate the Standard Error of the Mean ±SEM of 120 Samples (40x3) for Three Independent Experiments. The Symbols (*), (**) and (***) Indicate P< 0.05, P<0.01 and P<0.001, Respectively.

As can be seen in Fig. 2A, in vivo experiments, the initial level (control) of $^{45}Ca^{2+}$ uptake by brain tissue of young rats was 27% higher than in old ones. The injection with 10^{-9}M and 10^{-4}M ouabain led to the increase of $^{45}Ca^{2+}$ uptake both in young and old animals compared with control. However, 10^{-9}M ouabain-induced stimulation effect on $^{45}Ca^{2+}$ uptake in young animals was significantly higher (259%) than in old ones (162%), while in case of poisoning by 10^{-4}M ouabain, $^{45}Ca^{2+}$ uptake was higher in old animals (250%.) than in young ones (127%).

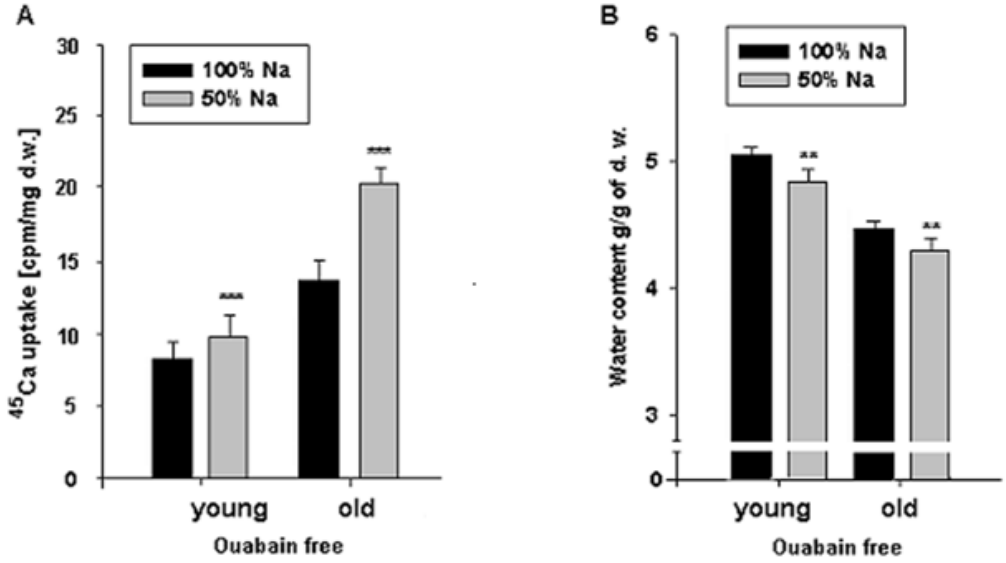
Unlike in vivo experiments, in vitro experiments, when brain slices were incubated in cold PS (30 min), the initial level of $^{45}Ca^{2+}$ uptake (control) was by 92% higher in old animals than in young ones (Fig. 2B). 10^{-9}M and 10^{-4}M ouabain concentrations also had age-dependent increasing effect on $^{45}Ca^{2+}$ uptake by brain samples. It is worth to note that the reciprocal effects of 10^{-9}M and 10^{-4}M ouabain on $^{45}Ca^{2+}$ uptake by tissue were more pronounced in old animals than in young ones (Fig. 2B). Furthermore, in 10^{-9}M ouabain-containing PS, $^{45}Ca^{2+}$ uptake by tissue had more pronounced age-dependent depressing character than $^{45}Ca^{2+}$ uptake by brain samples bathing in ouabain-free and 10^{-4}M ouabain-containing PS. However, this depressing effect was more pronounced in old animals than in young ones.

Ca^{2+} uptake by cells is realized by agonist- and potential-activated ionic channels and Na^+/Ca^{2+} exchange in reverse mode (R Na^+/Ca^{2+} exchange). It is known that the decrease of extracellular Na^+ ($[Na^+]_o$) leads to stimulation of R Na^+/Ca^{2+} exchange [42, 43]. Therefore, to check the contribution of R Na^+/Ca^{2+} exchange in $^{45}Ca^{2+}$ uptake as well as in cell hydration in norm and upon the effect of ouabain, age-dependent effects of different doses of ouabain on $^{45}Ca^{2+}$uptake, tissue hydration and [^{3}H]-ouabain binding with cell membranes in both PS containing 100% (normal) and 50% $[Na^+]_o$ and in vitro experiments were studied.

As can be seen in Fig. 3, 50% $[Na^+]_o$ PS led to activation of R Na^+/Ca^{2+} exchange in brain tissues (Fig. 3A), which was accompanied by tissue dehydration (Fig. 3B). Moreover, 50% $[Na^+]_o$ PS had stronger activation effect on R Na^+/Ca^{2+} exchange in brain tissue of old animals compared with young ones.

The activation effect of 10^{-9}M ouabain on R Na^+/Ca^{2+} exchange in brain tissue slices of young animals which was absent in ouabain-free PS, appeared at 50% $[Na^+]_o$ PS (Fig. 3C), like in vivo experiments (Fig. 2A). Furthermore, 10^{-4}M ouabain effect on R Na^+/Ca^{2+} exchange was insensitive to 50% $[Na^+]_o$ PS. In old animals 10^{-9}M ouabain had inhibition, while 10^{-4}M ouabain activation effect on R Na^+/Ca^{2+} exchange (Fig. 3D) at 50% $[Na^+]_o$ PS.

Previously we have shown that dose-dependent [^{3}H]-ouabain binding with a_3 isoform has down-regulation character in brain tissue of young animals which is absent in old ones because of age-dependent increase of $[Ca^{2+}]_i$ [30]. The comparative study of dose-dependent [^{3}H]-ouabain binding with cell membrane and its effect on tissue hydration in cortex samples bathing in 100% and 50% $[Na^+]_o$ PS both in young and old rats brings us to the same conclusion (Fig. 4).



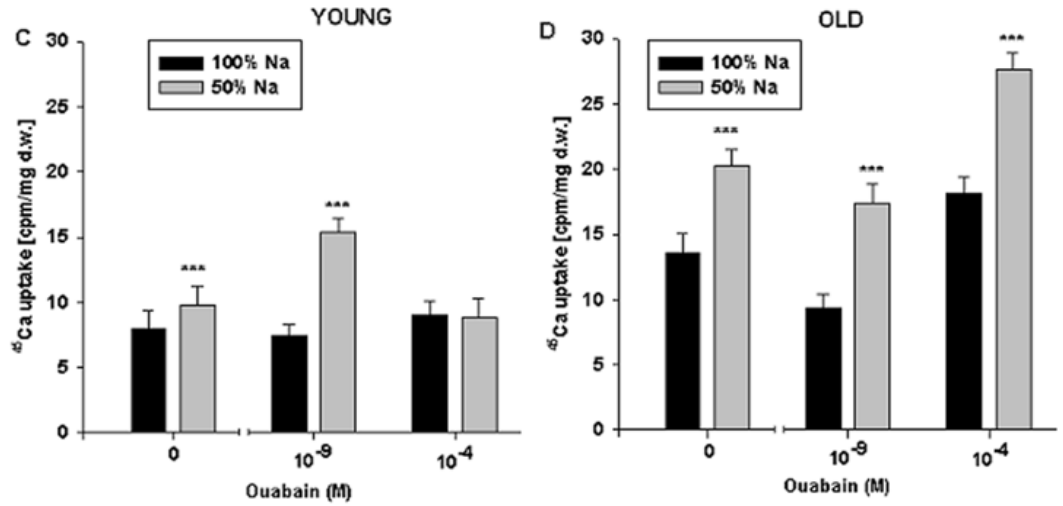


Fig. 3: Age-Dependent $^{45}Ca^{2+}$ Uptake and Water Content in Brain Cortex Tissues Incubated in 100% and 50% $[Na^+]_O$ PS. (A) $^{45}Ca^{2+}$ Content and Water Content (B) in Brain Tissue Samples of Young and Old Animals Incubated in 100% and 50% $[Na^+]_O$ PS Containing 11.2 Mci/L $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ Content in Brain Tissue Samples of Young (C) and Old (D) Animals Incubated in Ouabain-Free and Ouabain-Containing (10^{-9}M, 10^{-4}M) 100% and 50% $[Na^+]_O$ PS. Data on Tissue Hydration and $^{45}Ca^{2+}$ Uptake by Tissue at 100% $[Na^+]_O$ PS Were Taken from the Data Presented in Fig. 1 and Fig. 2, Respectively. Error Bars Indicate Standard Error of the Mean (±SEM) for Three Independent Experiments. The Symbols (**) and (***) Indicate P<0.01 and P<0.001, Respectively. For the Study of the Effect of 50% $[Na^+]_O$ PS on Tissue Hydration and $^{45}Ca^{2+}$ Uptake 45 Young and 45 Old Rats were Used.

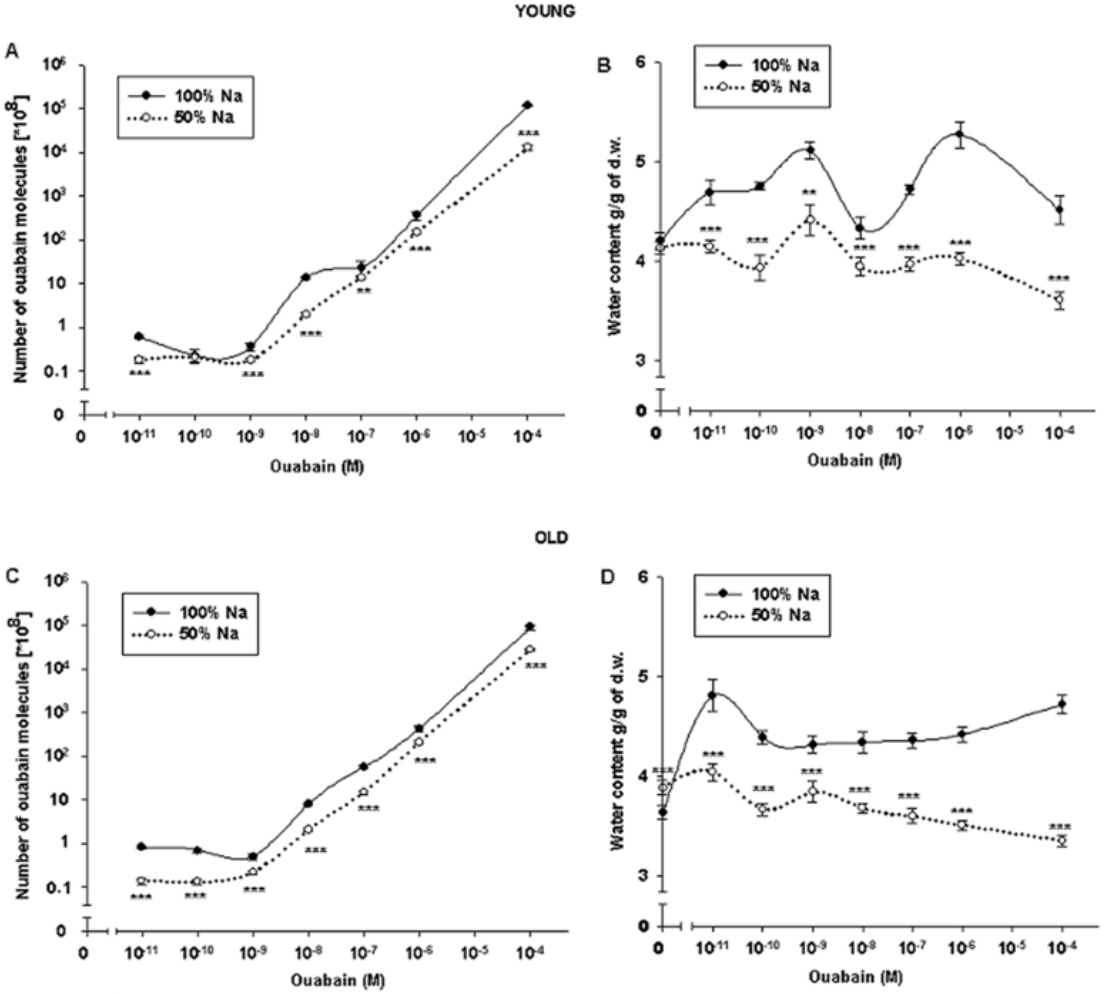


Fig. 4: Dose-Dependent $[^3H]$-Ouabain Binding with Cell Membrane and Its Effect on Water Content in Brain Tissue of Young (A) and Old (C) Animals after 30 Min of Intraperitoneal Injection of 100% (Continuous Lines) and 50% (Dash Lines) $[Na^+]_O$ PS (0.2 Ml/G of Animal Weight). Ordinates are Logarithmic and Define the Number of Ouabain Molecules $[X10^8]$ for G of D.W. of Tissues; Abscissas Indicate $[^3H]$-Ouabain Concentrations in M. Each Point of Curve Is the Mean ± SD for Three Independent Experiments (N=120). Dose-Dependent Ouabain Effect on Water Content (G/G of D.W.) of Brain Cortex Samples of Young (B) and Old (D) Rats Injected with 100% (Continuous Lines) and 50% (Dash Lines) $[Na^+]_O$ PS. Ordinates Indicate Mean Value of Water Content in Young (B) and Old (D) Animals; Abscissas Indicate $[^3H]$-Ouabain Concentrations in M. Error Bars Indicate the Standard Error of the Mean (±SEM) for Three Independent Experiments (N=120). The Symbols (**) and (***) Indicate P<0.01 and P<0.001, Respectively. 105 Young and 105 Old Rats have Been Used in These Series of Experiments.

As can be seen in Fig. 4, 50% $[Na^+]_o$ PS had strong depressing effect on ouabain binding with cell membrane in brain tissues of both young and old animals (Fig. 4, A and C). It is interesting to note that in young animals (Fig. 4A) the depressing effect of 50% $[Na^+]_o$ PS on dose-dependent down-regulation of $[^3H]$-ouabain binding with α_3 receptors was similar to aging effect on it (Fig. 4C). However, the depressing effect of 50% $[Na^+]_o$ PS on $[^3H]$-ouabain binding with α_3 receptors in brain tissue of old animals was more pronounced than in brain tissues of young ones.

The curves of dose-dependent ouabain effect on brain tissue hydration of young animals consisted of 6 components, 3 of which were located in zones of α_3 receptors (Fig. 4B), whereas, kinetic curve of dose-dependent $[^3H]$-ouabain binding with cell membrane in vivo experiments consisted of three family of receptors having different affinities [31]. All concentrations of ouabain at 50% $[Na^+]_o$ PS had dehydration effect on brain tissue and their dose-dependent curve in young animals at concentrations of 10^{-10}-10^{-4}M ouabain had parallel character at both 100% and 50% $[Na^+]_o$ PS. However, in case of $<10^{-10}$M ouabain, dose-dependent curves had opposite directions (Fig. 4B); at 100% $[Na^+]_o$ PS it had dose-dependent hydration, while at 50% $[Na^+]_o$ PS dose-dependent dehydration character (Fig. 4). In tissues of old rats in both 100 % and 50% $[Na^+]_o$ PS these curves had smoother character (Fig. 4D).

Age-dependent differences between the effects of low ($<10^{-9}$M) and high concentrations ($>10^{-4}$M) of ouabain on tissue hydration and R Na^+/Ca^{2+} exchange allow us to suggest that there are different mechanisms involved in this process. To check this suggestion dose-dependent ouabain effect on $^{45}Ca^{2+}$ efflux (F Na^+/Ca^{2+} exchange) from the cells of brain tissue was studied in the next series of experiments.

In order to estimate the role of $^{45}Ca^{2+}$ efflux in observed age-dependent changes of ouabain effects on the rate of R Na^+/Ca^{2+} exchange in brain tissue, the comparative study of 10^{-9}M and 10^{-4}M ouabain effects on $^{45}Ca^{2+}$ efflux from the preliminary $^{45}Ca^{2+}$-enriched brain samples were studied.

$[Ca^{2+}]_i$ can be decreased by removing Ca^{2+} from the cytosol by various mechanisms; a) by Ca^{2+}-pumps in membrane of endoplasmatic reticulum (ER) and in PM pushing Ca^{2+} into ER or outside the cell, respectively, b) by Na^+/Ca^{2+} exchange in forward mode (F Na^+/Ca^{2+} exchange) extruding cytosolic Ca^{2+} in exchange for extracellular Na^+. Ca^{2+}-pump in cell membrane controls the rest Ca^{2+} concentrations because of their high-affinity/low-capacity transport properties, whereas, Na^+/Ca^{2+} exchangers display low-affinity/high-capacity transport properties [44]. Therefore, in case of high $[Ca^{2+}]_i$, when Ca^{2+}-pumps in ER and PM are in inhibited state, the net Ca^{2+} efflux is mainly determined by F Na^+/Ca^{2+} exchange [45].

As can be seen in Fig. 5, the rate of $^{45}Ca^{2+}$ efflux from the brain samples of young rats was significantly higher than in old ones. Ouabain had inactivation effect on the rate of $^{45}Ca^{2+}$efflux from the brain samples of young rats, while in old animals ouabain had activation effect on it. It is worth to note that the inactivation effect of ouabain on $^{45}Ca^{2+}$ efflux from brain samples of young rats appeared at $<10^{-11}$M ouabain and was lessened by the increase of ouabain concentrations. However, activation effect of ouabain on $^{45}Ca^{2+}$ efflux from brain tissue samples in old animals was observed at $>10^{-11}$M ouabain. Furthermore, inactivation effect of nM ouabain on $^{45}Ca^{2+}$ efflux in young and activation effect on $^{45}Ca^{2+}$ efflux in old rats were significantly pronounced at 10^{-10} M concentration of ouabain (Fig. 4 and Fig. 5).

3.3. Ouabain effect on Ca^{2+} efflux from the cells

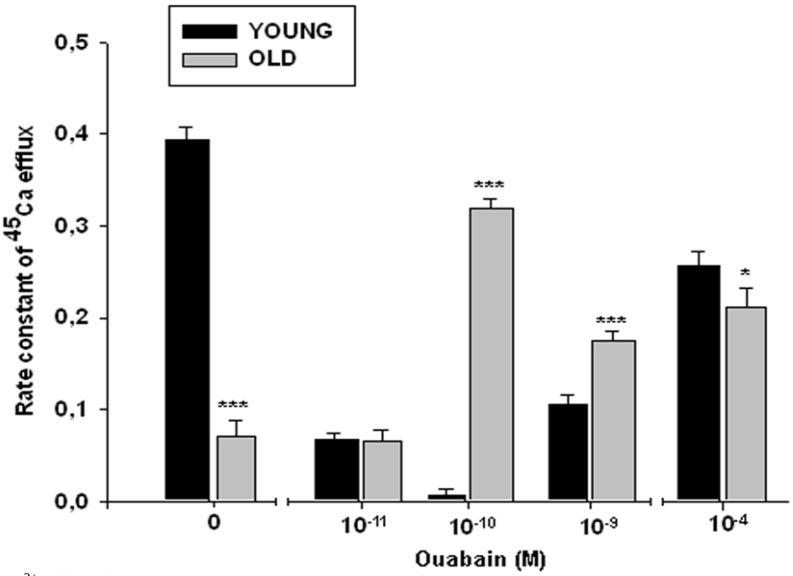


Fig. 5: The Rate Constant of $^{45}Ca^{2+}$ Efflux from Brain Cortex Tissue Samples of Young and Old Animals in Ouabain-Free and Ouabain-Containing (10^{-11}, 10^{-10}, 10^{-9}, 10^{-4} M) PS. Brain Slices were Preliminary Enriched by $^{45}Ca^{2+}$ by Incubating them for 60 Min in K^+-Free (Containing 50% Nacl) Cold (7°c) PS (Containing1.8 Ml $^{45}Ca^{2+}$). Error Bars Indicate the Standard Error of the Mean (±SEM) for Three Independent Experiments (N=120). The Symbols (*), and (***) Indicate P<0.05 and P<0.001, Respectively. 75 Young and 75 Old Rats were used in these Series of Experiments.

4. Discussion

Traditionally, metabolic regulation of cell hydration is explained either by the changes of transporting function of membrane [46], [47], [48], [21], [22] or by the changes of sorption properties of cytoplasm [49], [50], [51]. However, there is a lack of adequate consideration to fundamental role of intracellular signaling systems controlling the release of endogenous H_2O in regulation of cell hydration. It is known that 42 H_2O molecules are formed in cytoplasm during oxidation of one molecule glucose [26]. As the rate of oxidation-phosphorylation process depends on the intensity of ATP utilization by transporting membrane ATPases, it may be predicted that the release of endogenous H_2O in cytoplasm will be activated in response to the increase of membrane permeability. Therefore, neuronal swelling as a result of the increase of its membrane permeability will lead to stimulation of membrane ATPases which in its turn will bring to generation of endogenous H_2O. The latter will reverse H_2O influx into its efflux through the membrane having inhibitory effect on inward ionic currents in membrane [52], [53], [54]. Therefore, age-dependent cell dehydration (Fig. 1) can be considered as a result of depression of intracel-

lular oxidative processes. This suggestion is supported by the data that initial level of tissue hydration of young and old rats was higher in vitro experiments (Fig. 1B) than in vivo ones (Fig. 1A). It is obvious that cell membrane permeability in brain tissue samples was higher in vitro experiments than in vivo ones which predicted the increase of ATP utilization for controlling intracellular homeostasis. The data presented in Figure 1A clearly indicate that age-dependent elevation effect of 10^{-4}M ouabain on cortex tissue hydration cannot be explained only by 10^{-4}M ouabain-induced inhibition of ion-transporting function of Na^+/K^+-ATPase in plasma membrane (PM). Therefore, cell hydration in response of the increase of cell membrane permeability can be considered as a result of the uptake of osmotic particles by cell as well as the increase of endogenous H_2O. The suggestion that cell hydration depends on its metabolic activity is also supported by the fact that in vitro experiments age-dependent tissue hydration disappeared at 10^{-4}M ouabain-induced inhibition of Na^+/K^+ pump activity (Fig. 1B) which was observed in vivo experiments (Fig. 1A).

Na^+/K^+ pump inhibition-induced cell swelling [55], [17], [18], [19], [22] has been traditionally explained by the depression of its ion-transporting function in membrane. From this point of view as transporting function of Na^+/K^+-pump is depressed by aging, it is predicted that 10^{-4}M ouabain-induced Na^+/K^+-pump inhibition [56] could have more pronounced effect on cell hydration in brain of young animals than in brain of old ones. However, in present work the opposite effect was observed which could serve as strong evidence that 10^{-4}M ouabain, besides inhibition effect on Na^+/K^+-pump, has also depressing effect on oxidation-induced release of water molecules in cytoplasm which has age-dependent weakening character (Fig. 1). At present, it is well established that among three isoforms of Na^+/K^+-ATPase, α_1 (fully) and α_2 (partly) have ion-transporting functions, while α_3 isoform, activated by $\leq 10^{-9}$M ouabain, performs only intracellular signaling function [45], [57], [58]. Although the crucial role of Na^+/K^+-pump in regulation of neuronal hydration is well established [17], [18], [13], [8], [10], [21], [22], the individual roles of different isoforms of Na^+/K^+-ATPase (working molecules of pump) in this process is not elucidated yet.

It is known that brain tissue consists of neuronal and glial cells. Therefore, brain tissue hydration is a sum of neuronal and glial cells hydration. As it was mentioned above, Na^+/K^+-ATPase in cell membrane has ion-transporting and intracellular signaling functions. Moreover, α_3 isoform of Na^+/K^+-ATPase which is localized only in neurons has only signaling function. Whereas, α_1 and α_2 are localized in glial cells and have transporting and signaling functions [59], [60], [61], [62], [63]. Therefore, the effect of $<10^{-9}$M ouabain on brain cortex tissue hydration can be considered as a result of activation of a_3 isoform in neuronal membrane.

The fact that in vivo experiments 10^{-9}M ouabain had stronger activation effect on tissue hydration than 10^{-4}M ouabain (Fig. 1A) allows us to suggest that 10^{-9}M ouabain-induced activation of intracellular signaling system brings to activation of endogenous H_2O in cytoplasm. This suggestion is supported by the data on the disappearance of 10^{-9}M ouabain-induced increase of tissue hydration in vitro experiments, where metabolic activity of tissue was depressed in cold (7°C) PS, while 10^{-4}M ouabain-induced tissue hydration was increased (Fig. 1B) compared with the hydration of the samples incubated in ouabain-free PS.

The data presented in Figure 2 show that the injection of 10^{-9}M ouabain leads to the increase of $^{45}Ca^{2+}$ uptake by cells, which has an age-dependent weakening character (Fig. 2 A). The data that the effect of 10^{-9}M ouabain on $^{45}Ca^{2+}$ uptake is significantly higher than 10^{-4}M ouabain (having strong inhibition effect on Na^+/K^+-pump) clearly indicate that 10^{-9} M ouabain-induced activation of $^{45}Ca^{2+}$ uptake cannot be explained by the inhibition of Na^+/K^+-pump as it is traditionally suggested [42], [45].

It is known that $^{45}Ca^{2+}$ uptake by cells could be realized by agonist-, potential-activated Ca^{2+}-channels and R Na^+/Ca^{2+} exchange in membrane. In our previous study it has been shown that low ($<10^{-7}$M) concentrations of ouabain has no effect on Na^+/K^+-pump activity [8], instead it has activation effect on cAMP formation and R Na^+/Ca^{2+} exchange [64]. This allows us to suggest that 10^{-9} M ouabain-induced activation of $^{45}Ca^{2+}$ uptake (Fig. 2, A) is due to the activation of R Na^+/Ca^{2+} exchange.

The activation of $^{45}Ca^{2+}$ uptake by brain tissue samples in vitro experiments can be explained by activation of ionic channels and R Na^+/Ca^{2+} exchange. Therefore, the age-dependent elevation effect of $^{45}Ca^{2+}$ uptake in vitro experiments compared with that of in vivo ones can be explained by the depression of $[Ca^{2+}]_i$ buffering system in old animals.

As 10^{-4}M ouabain has inhibitory effect on Na^+/K^+-pump [56] which leads to membrane depolarization and excitation [65, 66], $^{45}Ca^{2+}$ uptake can be considered as a result of activation of Ca^{2+} channels and R Na^+/Ca^{2+} exchange (Fig. 2). The data that in vivo experiments the activation of $^{45}Ca^{2+}$ uptake and its age-dependent reverse took place at 10^{-4}M ouabain-induced pump inhibition (Fig. 2A), which was more significant than in vitro experiments (Fig. 2B), indicate the crucial role of Na^+/K^+-pump in metabolic controlling of $[Ca^{2+}]_i$ buffering system in brain cells. As it was noted above, 10^{-9}M ouabain-induced activation of $^{45}Ca^{2+}$ uptake can be considered as a response of activation of α_3 isoform having only signaling function [57], [63]. The fact that 10^{-9}M and 10^{-4}M ouabain-induced activation effects on $^{45}Ca^{2+}$ uptake are realized by different mechanisms is supported by the following experimental data: a) in vivo experiments 10^{-9}M ouabain-induced activation of $^{45}Ca^{2+}$ uptake in young animals was more pronounced than 10^{-4}M ouabain effect, while 10^{-4}M ouabain stimulation effect in old animals was more pronounced than 10^{-9}M ouabain effect (Fig. 2A), b) in vitro experiments 10^{-9}M ouabain had depressing effect on $^{45}Ca^{2+}$ uptake by brain tissue samples both in young and old animals compared with ouabain-free PS, while 10^{-4}M ouabain had more pronounced activation effect on $^{45}Ca^{2+}$ uptake both in young and old animals than 10^{-9}M ouabain (Fig. 2B).

As Na^+/Ca^{2+} exchanger functions in stoichiometry of $3Na^+:1Ca^{2+}$ and in case of K^+-dependent Na^+/Ca^{2+} exchanger in stoichiometry of $4Na^+:1Ca^{2+}+1K^+$ [64], it was predicted that 10^{-9}M ouabain-induced activation of R Na^+/Ca^{2+} exchange should have dehydration effect on brain tissue, however, hydration effect was observed (Fig. 1A). From these data it can be concluded that there is a common metabolic mechanism localized in cytoplasm which is responsible for 10^{-9}M ouabain-induced activation of R Na^+/Ca^{2+} exchange and tissue hydration. This metabolic mechanism has age-dependent dysfunctional character (Fig. 2A) and is depressed in vitro experiments when metabolic activity is inhibited (Fig. 2B). This suggestion is confirmed by the data of the comparative study of dose-dependent ouabain effects on cell hydration and R Na^+/Ca^2 exchange in brain tissue samples bathing at 100% and 50% $[Na^+]_o$ PS. As it is known that the decrease of $[Na^+]_o$ brings to the activation of R Na^+/Ca^2 exchange [42], 50% $[Na^+]_o$ PS-induced activation of R Na^+/Ca^{2+} exchange was accompanied by cell dehydration (Fig. 3). It is known that there is an age-dependent increase of $[Ca^{2+}]_i$ [39] which predicts the decrease of 50% $[Na^+]_o$ PS-induced R Na^+/Ca^2 exchange. However, in our experiments the opposite effect was observed. Though the initial level of $[Ca^{2+}]_i$ in old animals was higher than in young ones, activation effect of 50% $[Na^+]_o$ PS on R Na^+/Ca^{2+} exchange in old animals was more pronounced than in young ones (Fig. 3A). This can be explained by the existence of an intracellular mechanism controlling $[Ca^{2+}]_i$, such as Ca^{2+}-Calmodulin-NO-GMP cascade [67] having activation effect on F Na^+/Ca^2 exchange [68]. This activation effect is stronger in the brain neurons of young rats than in brain neurons of old ones.

The existing modulation effects of 10^{-9}M and 10^{-4}M ouabain on R Na^+/Ca^2 as well as on tissue hydration at both 100% and 50% $[Na^+]_o$ PS (Fig. 3, C and D, Fig. 4, B and D) indicate that the mechanism responsible for 10^{-9}M and 10^{-4}M ouabain-induced increase of cell hydration and rate of R Na^+/Ca^{2+} has cytoplasmic origin. This suggestion is supported by the data on 10^{-9}M ouabain-induced reactivation of R Na^+/Ca^{2+} exchange in brain tissue of young animals in vivo experiments (Fig. 2A) at 50% $[Na^+]_o$ PS which was reversed in normal PS in vitro experiments (Fig. 3C). Moreover, the effect of 10^{-4}M ouabain on $^{45}Ca^{2+}$ uptake by tissue

of old animals bathing in 50% $[Na^+]_o$ PS (Fig. 3D) serves as additional evidence supporting the above mentioned suggestion.

Considering the fact that the differences of ionic gradients of Na^+/Ca^{2+} ions on membrane serves as energy sources for Na^+/Ca^{2+} exchange, it becomes clear that the activation of cytoplasm absorption of $[Ca^{2+}]_i$ will attenuate 50% $[Na^+]_o$ PS-induced activation of R Na^+/Ca^{2+} exchange. Therefore, 10^{-9}M ouabain-induced reactivation of R Na^+/Ca^{2+} at 50% $[Na^+]_o$ PS in neurons of young animals could be an evidence for 10^{-9}M ouabain-induced activation of $[Ca^{2+}]_i$ sorption properties of intracellular structures. The fact that the mentioned effect of 10^{-9}M ouabain was depressed in brain neurons of old rats (Fig. 2) can be explained by the age-dependent increase of $[Ca^{2+}]_i$.

The decrease of [^{3}H]-ouabain binding with cell membrane at 50% $[Na^+]_0$ PS in young and old animals compared with those at 100% $[Na^+]_0$ PS is a result of the increase of $[Ca^{2+}]_i$ (Fig. 5, A and C). This effect which was more pronounced at [^{3}H]-ouabain binding with a_3 receptors than with a_2 and a_1 is in harmony with literature data that among mentioned isoforms, a_3 has higher affinity to Ca^{2+} than a_2 and a_1 [45], [57]. The age-dependent increase of 50% $[Na^+]_o$-induced inhibition of [^{3}H]-ouabain binding can be explained by the depression of $[Ca^{2+}]_i$ buffering properties of cytoplasm. The data on the comparative study of dose-dependent ouabain effects on tissue hydration of young and old animals in 100% and 50% $[Na^+]_o$ PS bring us to the same conclusion (Fig. 4, B and D). As it was predicted, 50% $[Na^+]_o$-induced activation of R Na^+/Ca^{2+} exchange led to brain tissue dehydration at all ouabain concentrations both in young and old animals. The obtained data demonstrated pronounced age-dependent character of kinetics of ouabain effects on tissue hydration; in young animals these curves consisted of 6 components, whereas, in old animals they had 3 components. Multi-component character of the curves of dose-dependent ouabain effects on tissue hydration in young animals which was depressed by aging probably can be explained by the existence of caveolae containing aquaporins [10], especially, by aquaporin-4 (AQP4) which is one of the most abundant molecules in brain tissue [69]. This suggestion cannot be final and needs more detailed investigation.

The suggestion that there is an intracellular signaling system controlling $[Ca^{2+}]_i$ absorption properties of cytoplasm which is sensitive to extremely low concentrations of ouabain ($\leq 10^{-9}$M) is supported by the data on dose-dependent effect of ouabain on $^{45}Ca^{2+}$ efflux from the cortex tissue. As it was noted above, in case of high $[Ca^{2+}]_i$, when Ca^{2+}-pumps in ER and PM are in inhibited state, the net $^{45}Ca^{2+}$ efflux is mainly determined by F Na^+/Ca^{2+} exchange [43]. Therefore, in vitro experiment when $[Ca^{2+}]_i$ is high, $^{45}Ca^{2+}$ efflux can also be considered as a result of activation of F Na^+/Ca^{2+} exchange.

In vitro experiments ouabain had inactivation effect on $^{45}Ca^{2+}$ efflux from brain tissue of young animals, while it had activation effect in old animals (Fig. 5). The threshold of inactivation effect of ouabain on $^{45}Ca^{2+}$ efflux was much lower ($<10^{-11}$M) in young animals than the threshold ($>10^{-10}$M) of its activation effect on Ca^{2+}efflux in old ones (Fig. 5). These data clearly indicate that different intracellular signaling systems are responsible for ouabain-induced inhibition and activation of $^{45}Ca^{2+}$ efflux in young and older animals, respectively. This suggestion is supported by the data that the curves of dose-dependent ouabain effect on $^{45}Ca^{2+}$ efflux both in young and old animals had opposite character at ouabain concentrations of $<10^{-8}$M (a_3 receptors), while they had parallel character at high concentrations ($>10^{-8}$M). These data are in harmony with literature [63] and our previous data that a_3 receptors have more pronounced age-dependent character than a_2 and a_1 because of its high affinity to $[Ca^{2+}]_i$ [30].

The non-monotonic curve of ouabain dose-dependent effect on $^{45}Ca^{2+}$ efflux in the study of brain tissue hydration of young rats (Fig. 4) can be explained by the existence of caveolae in membrane containing different signaling proteins [10] in result of which the changes of cell volume have a discreet character [8]. The absence of correlation between the rate of F Na^+/Ca^{2+} exchange and cell hydration (instead of predicted hydration) one more time witnesses that the age-induced cell dehydration cannot be a direct result of depression of F Na^+/Ca^{2+} exchange.

Thus, the obtained data allow us to conclude that the dysfunction of intracellular signaling system controlling cell hydration plays a primary role, while ion-transporting cell membrane mechanisms have a secondary role in age-induced brain tissue dehydration.

Based on the data of our previous work that nM ouabain-induced activation of R Na^+/Ca^{2+} exchange is accompanied by the increase of intracellular contents of cAMP [64], which stimulates Ca^{2+}-pump in ER membrane [41], we suggest that the decrease of $[Ca^{2+}]_i$ brings to the activation of R Na^+/Ca^{2+}exchange (Fig. 2). The nM ouabain-induced cell hydration can be explained by Ca^{2+}-ATPase products (ADP+P)-induced activation of ATP-synthesis which is accompanied by the formation of endogenous H_2O in cytoplasm. Besides, it is suggested that there is another pathway through ER activates mitochondrial function. It demonstrates the close contact between ER and mitochondria that is involved in maintaining a dynamic cross-talk between the two organelles [70]. ER-mitochondrial cross-talk is fundamental for the up-regulation of mitochondrial metabolism in stimulated cells and plays a key role in decoding Ca^{2+}-mediated apoptotic signals [71].

It is known that in old animals because of high $[Ca^{2+}]_i$, lipase C activity in membrane is increased. Previously it has been shown that there is a negative correlation between Na^+/K^+-ATPase activity and intracellular cAMP content and this correlation disappears upon the effect of lipase activation in membrane [72]. Therefore, it is suggested that ≤nM ouabain through stimulation of phosphatidylinositol cycle could lead to activation of inositol 1,4,5-trisphosphate ($InsP_3$)-induced Ca^{2+} efflux from ER and potential-dependent L-Ca^{2+} channels as a result of which $[Ca^{2+}]_i$-Calmodulin-NO-cGMP-Ca^{2+}efflux cascade is stimulated [67]. The latter has an activation effect on F Na^+/Ca^{2+} exchange in cell membrane [68]. This suggestion is not final and needs more detailed investigation.

Thus, the obtained data allow us to suggest that the dysfunction of a_3 isoform-dependent signaling system controlling cell hydration is a primary mechanism for generation of age-dependent neuronal dehydration and increase of $[Ca^{2+}]_i$.

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