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Aging Leads to Over-Expression of Na^+/K^+ Pump Units in Liver and Na^+/Ca^{2+} Exchangers in Brain Cortex

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Abstract

The metabolic controlling of tissue hydration is the fundamental parameter determining cell functional activity and its dysfunction is the common consequence of any cell pathology, including aging. The aim of the present study is to reveal the differences of age-dependent metabolic controlling of cell hydration of excitable tissue such as brain cortex and non-excitable tissues such as liver and spleen. For this purpose, the age-dependent sensitivity of cell hydration in excitable and non-excitablet issues is studied through depressing metabolic activity by cooling and its activation by supplying animals with distilled water, by inactivation of Na^+/K^+ pump and activation of Na^+/Ca^{2+} exchange in the reverse mode. The obtained data bring us to the conclusion that the metabolic regulation of cell hydration in excitable tissue is realized by the activation of electrogenic Na^+/K^+ pump and Na^+/Ca^{2+} exchange in the cell membrane and the formation of endogen water by mitochondrial activity, while the regulation of cell hydration in non-excitable tissue is carried out only by the activity of mitochondrial function. Aging leads to an over-expression of Na^+/K^+ pump units in liver and Na^+/Ca^{2+} exchanger in brain cortex of rats.

Keywords

Rat, Hydration, Brain Cortex, Liver, Spleen, [^{3}H]-Ouabain

1. Introduction

Intracellular water, being the dominant component of cell and the common medium for intracellular metabolic reactions, has a determined role in the regulation of cell activity realizing by surface-dependent changes in the number of

functional active proteins in the membrane having enzyme, receptor, ionic channel properties [1] and hydration-dependent stimulation of intracellular macromolecules' activity [2]. Therefore, the dysfunction of metabolic controlling of cell hydration can be considered a common consequence of cell pathology, including aging. It is known that aging-induced dehydration of excitable tissues (ET), like brain cortex and heart muscle, leads to cell apoptosis or necrosis [3]. Although the non-excitable tissues (NET) such as liver and spleen are dehydrated in aging the dysfunction of their metabolic activity very often brings to over-hydration and increase of cell proliferation [4] [5]. It is also known that the membrane of NET cells is highly permeable for water molecules than for ions and has more pronounced volume of recovering mechanism than that in ET cells [5] [6].

Our previous study has shown that the metabolic water efflux from the cells serves as the key mechanism for controlling the low permeability of inward ionic currents through the membrane [7]. The dysfunction of Na^+/K^+ pump, being the common consequence of cell pathology, including aging can be considered as the main reason for age-dependent tissue dehydration. It is known that Na^+/K^+ pump generating Na^+ gradient on the membrane serves as the energy source for Na^+/Ca^{2+} and Na^+/H^+ exchangers in the membrane. As the cell membrane of NET is highly permeable for water as well as for H^+, the electrogenic properties of the Na^+/K^+ pump and Na^+/Ca^{2+} exchange are shunted by H^+ influx. So, it is suggested that Na^+/K^+ pump and Na^+/Ca^{2+} exchange are unable to generate the osmotic gradients on NET cells of the membrane, while in ET cell membrane they generate these gradients. Therefore, it is predicted that in the regulation of cell volume in ET cells involve the electrogenic ionic transporting mechanisms in the membrane and mitochondrial processes generating the water molecule release in cytoplasm. To check this hypothesis in the present work, the comparative study of the role of the membrane ion transporting mechanisms and the intracellular oxidative processes in age-dependent impairment of metabolic controlling of cell hydration in ET and NET in *in vivo* and in *in vitro* experiments are performed using different experimental conditions.

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).

The experiments were performed on healthy young (6 weeks old, weighting ~35 g) and old (18 months old, weighting ~250 g) albino rats. They were regularly examined, kept under control of the veterinarians in LSIPEC and reserved in a specific pathogen-free animal room under optimum conditions of 12 h light/dark cycles, at temperature of 22˚C ± 2˚C, with 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 $NaHCO_3$, and 0.42 NaH_2PO_4 and adjusted to pH 7.4 was used. PS with 50% of NaCl was received by replacing 68.5 mM of NaCl from 137 mM NaCl with 2 M mannitol dissolved in PS for maintaining the osmolarity of the solution. These two types of PS in corresponding figures named as 100% Na and 50% Na. All chemicals were obtained from "Medisar" Industrial Chemical Importation Company (Yerevan, Armenia). The distilled water (DW) was received in laboratory by means of corresponding apparatus. The [^{3}H]-ouabain with specific activity 25.34 Ci/mM (PerkinElmer, Massachusetts, USA) at 10^{-4}M, 10^{-9}M and cold (non-radioactive) ouabain at 10^{-4}M, 10^{-9}M were used for incubation and intraperitoneal (i/p) injections. The volume of injected solutions was adjusted according to the weight of animals (0.02 ml/g).

2.3. Tissue Preparation

The experimental data were received in *in vivo* and in *in vitro* conditions. The tissue samples from each experiment were investigated after decapitation. Since the anesthetics with different chemical and pharmacological profiles significantly affect the metabolic processes in tissues [8], [9] in our experiments the animals were sharply immobilized by freezing method [10] and decapitated. Full absence of somatic reflexes was recorded after this procedure. The brain cortex, liver, spleen tissues were isolated and dissected according to the corresponding experiments.

2.4. Experimental Design

The experiments of the first, second and third series were carried out in *in vivo* conditions. In each animal group (young and old) 6 rats were taken in all series of experiments. In the first series of experiments the animals of both young (n = 6) and old (n = 6) subgroups were i/p injected by PS (as a control). After 30 min the animals were decapitated and 5 samples were taken from each animal's brain cortex, liver and spleen tissues. Thus, from each tissue were received 30 samples of brain cortex, liver and spleen. They were divided into 2 parts (15 samples in each), which incubated for 30 min in PS at 20˚C and 7˚C, respectively. In experiments described below the tissue samples were taken after animal decapitation.

For the second series of experiments the data of control group (the tissue samples of which were incubated in PS at 20˚C) were compared with the corresponding data of young as well as of old experimental animal groups being i/p injected with [^{3}H]-ouabain at 10^{-4}M. In these experimental groups 3 young and 3 old rats were taken and the number of samples from each investigated tissue was the same as it is mentioned above.

The third series of experiments were carried out like the second one but the animals of experimental groups were i/p injected with [^{3}H]-ouabain at 10^{-9}M. The obtained data were compared with the same data of control group as in the

second series.

The 4th-6th series of experiments were made in *in vitro* conditions. From 6 animals of each group (young and old) 5 samples of each tissue were taken. So, 30 samples from each tissue were divided into 2 parts (15 samples in each), which were incubated for 30 minutes in the two types of PS (100% Na and 50% Na) in addition to them the radioactive Ca ($^{45}Ca^{2+}$). The 5th and 6th series were performed in the same way as the 4th one and in each corresponding PS (with ^{45}Ca) where incubated their tissue samples were also added the non-radioactive ouabain at 10^{-4}M (5th series) and 10^{-9}M (6th series), respectively.

The 7th-9th series of experiments were carried out on animals drinking (for 10 days) the distilled water (DW) and after being i/p injected by PS. In each series 3 young and 3 old animals were taken. The obtained data from 7th series were compared with that received in the 1st series (data from tissue samples of animals were incubated in PS at 20˚C). After 10 days of drinking DW the animals of 8th and 9th series were being i/p injected by [^{3}H]-ouabain at 10^{-4}M (8th series) and 10^{-9}M (9th series), respectively. The results of these experiments were compared with the corresponding data received in 2nd and 3rd series, respectively.

2.5. Definition of Water Content of Brain Tissues

The water content of brain cortex, liver and spleen tissues was determined by traditional "tissue drying" method [11]. After measuring the wet weight (w.w.) of tissue samples they were 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 1 g of d.w. tissue was counted by the following equation: (w.w.–d.w.)/d.w.

2.6. Counting of [^{3}H]-Ouabain Molecules

The tissue samples in *in vivo* experiments, which were subjected to [^{3}H]-ouabain, were homogenized in 50 µl of 68% HNO_3 solution after determination of wet and dry weights. Then 2 ml of Bray's scintillation fluid was added and chemo luminescence of samples was quantified with 1450-Micro Beta liquid scintillation counter (Wallac, Turku, Finland). The number of [^{3}H]-ouabain molecules' binding with cell membranes was defined per mg of dry weight of samples. The same procedure (the definition of number of [^{3}H]-ouabain molecules) was performed on the tissue samples from in *in vitro* experiments after removing them from the oven and determining their water content.

2.7. Measurement of $^{45}Ca^{2+}$ Uptake

PS with the radioactive $^{45}Ca^{2+}$ (PerkinElmer, Massachusetts, USA) was received by substituting 0.0115 mM of $CaCl_2$ from 1.8 mM $CaCl_2$ by radioactive one (with 11.2 mCi/l activity). The measurement of $^{45}Ca^{2+}$ uptake was carried out as in the case of definition of [^{3}H]-ouabain molecules. The quantity of $^{45}Ca^{2+}$ in tissue slices was expressed by cpm/mg d. w.

2.8. Statistical Analysis

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

3. Results

It is known that aging is connected with the dysfunction of metabolic processes in the living objects. In order to find out the difference between the metabolic sensitivity of tissues, the effect of cold (7˚C) PS on ET and NET hydration of young and old animals are studied. As can be seen in **Figure 1**, in the group of young as well as old animals i/p injected by PS, the incubation of brain cortex tissue samples in cold PS lead to significant dehydration (**Figure 1(a)**), while in liver and spleen tissues it brings to pronounced opposite effect, *i.e.* to over-hydration (**Figure 1(b)** and **Figure 1(c)**).

It is worth to note that the temperature-dependent tissue hydration has more pronounced age-dependent increasing character in the brain and liver tissues (**Figure 1(a)** and **Figure 1(b)**), while in the spleen tissues the temperature sensitivity of tissue hydration is higher in young animals than in the old ones (**Figure 1(c)**).

The previous research data have established the key role of Na^+/K^+ pump in the regulation of cell volume [1] [7] [12]. To estimate the age-dependent effect of Na^+/K^+ pump on cell hydration and ouabain binding in the second series of experiments the effect of i/p injected [^{3}H]-ouabain at 10^{-4}M in ET and NET is studied. The received data have been compared with those of animal group i/p injected by PS (data of black bars in **Figure 1**). At first sight, the opposite results of water content can be noted in ET (brain cortex) and NET (liver, spleen) in young as well as in old animals, *i.e.* the increase of water content (**Figure 2(a)**)

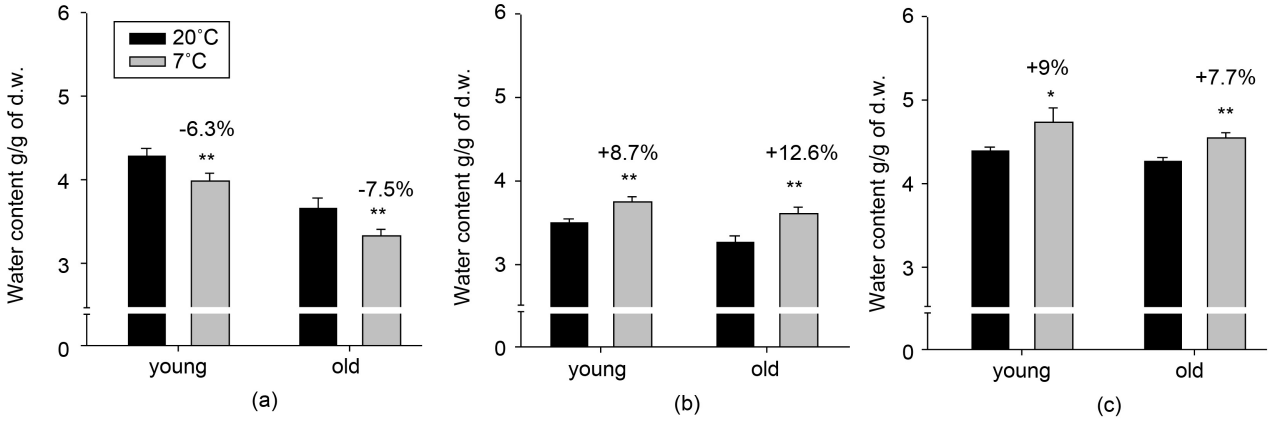

Figure 1. The variation of water content in brain cortex, liver and spleen tissue samples after their incubation in PS at 20˚C (black bars) and 7˚C (gray bars), respectively. Each bar represents the mean ± SEM (n = 45). The symbols (*), (**) indicate $p < 0.05$ and $p < 0.01$, respectively. The numbers in % indicate the difference between levels of hydration in each group. All data were obtained from three independent experiments.

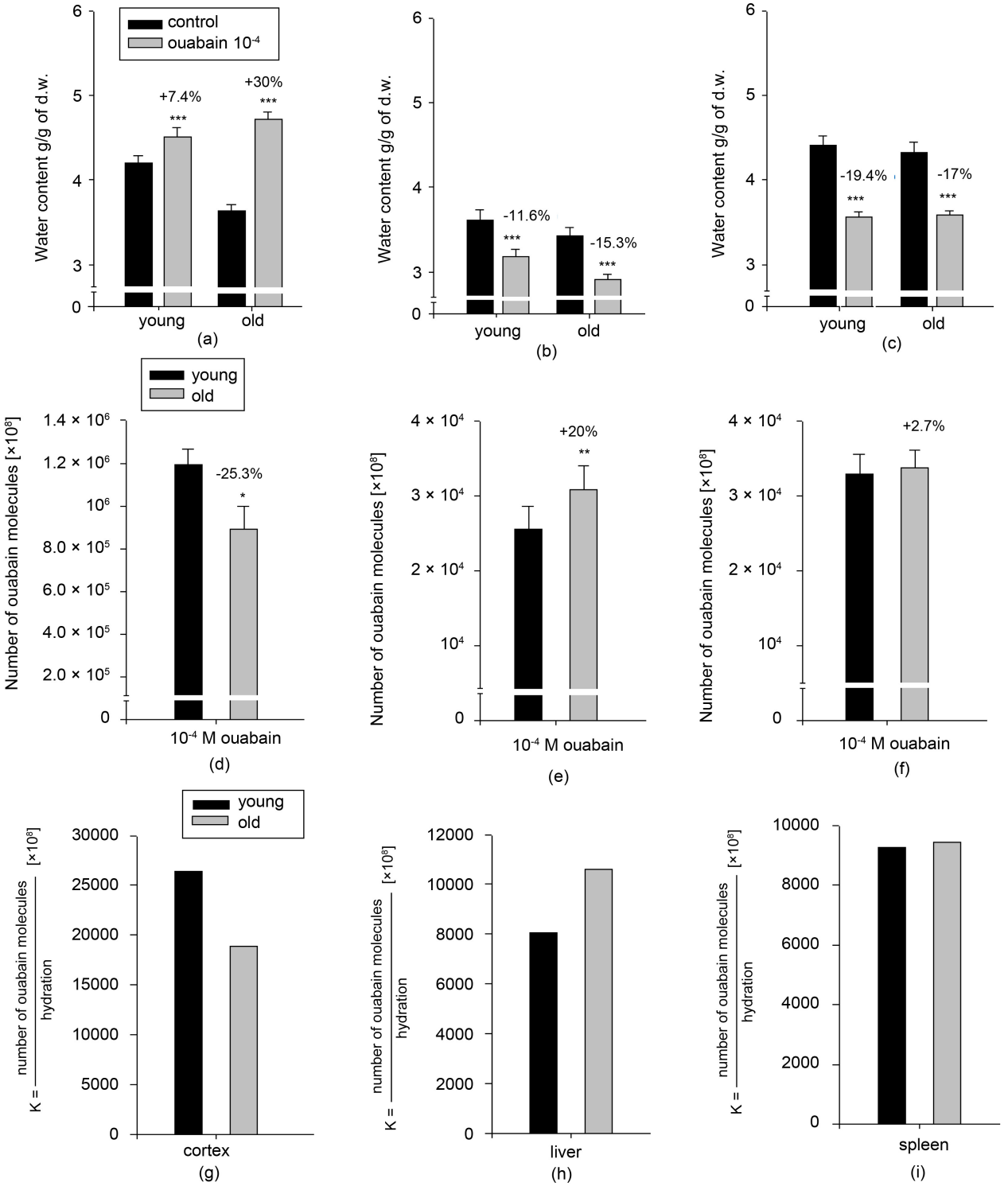

Figure 2. The effect of [^{3}H]-ouabain at 10^{-4}M on water content and number of ouabain molecules in brain cortex, liver and spleen tissues. Black bars on (a)-(c) indicate the mean value of water content in the tissues of control animal group (i/p injected by PS). Gray bars on (a)-(c) indicate the mean value of water content in the tissues of experimental animal group (i/p injected by [^{3}H]-ouabain at 10^{-4}M). Each bar represents the mean ± SEM (n = 45). (d)-(f) demonstrate the number of ouabain molecules in young (black bars) and old (gray bars) animal tissues. The numbers in % indicate the difference between levels of hydration and the number of ouabain molecules. (g)-(i) indicate the ratio between the number of ouabain molecules and the level of water content. The symbol (***) indicates $p < 0.005$. All the data were obtained from three independent experiments.

in ET and its decrease in NET (**Figure 2(b)**, **Figure 2(c)**). As can be seen in **Figure 2(a)**, the over-hydration is more pronounced in the brain cortex tissue of old animals than in young ones. The effect of dehydration in liver tissue of old animals (**Figure 2(b)**) is higher than that in young animals, while in spleen tissue the percent of dehydration is higher in case of young animals in comparison to the old ones (**Figure 2(c)**). From these data it is suggested that on the basis of cell hydration in ET and NET lay different mechanisms.

In spite of the fact that [^{3}H]-ouabain at 10^{-4}M in ET of old animals leads to more expressed over-hydration than that in young ones, the number of ouabain molecules in cortex tissue of old animals is lower than in young ones (**Figure 2(d)**). As for the ouabain binding in NET (**Figure 2(e)**, **Figure 2(f)**), it must be noted that the number of ouabain molecules in old animal tissues is higher than in young ones. The coefficients reflecting the ratio between the number of ouabain molecules and cell hydration (**Figure 2(g)**, **Figure 2(h)**) are similar to those of corresponding ratio between their numbers of ouabain molecules (**Figure 2(d)**, **Figure 2(f)**).

The previous data [13] have observed that nM ouabain stimulates Na^+/Ca^{2+} exchange in the reverse (R) mode and leads to age-dependent weakening of hydration effect in ET. To find out the role of RNa^+/Ca^{2+} exchange in the regulation of NET cell hydration and its age-dependency, in the next (3rd) series of experiments the comparative study of i/p injection of nM[^{3}H]-ouabain is studied. As in case of the abovementioned experiments (**Figure 2**), the injection of nM [^{3}H]-ouabain brings to the same results: the increase of water content in ET (**Figure 3(a)**) and its decrease in NET (**Figure 3(b)**, **Figure 3(c)**). As can be seen in **Figure 3(a)**, the over-hydration in ET of young animals is higher than that in ET of old ones, but the number of ouabain molecules in ET of old animals is higher than that in ET of young ones (**Figure 3(d)**). The ouabain binding in NET (**Figure 3(e)**, **Figure 3(f)**) demonstrates the correlation between the level of hydration and the number of ouabain molecules. Clearer correlation between the number of ouabain molecules binding with membrane and its cell hydration can be detected by means of the coefficients determined their relationship (**Figure 3(g)**, **Figure 3(i)**).

It is known that the decrease of Na gradient on the cell membrane leads to the activation of RNa^+/Ca^{2+} exchange [14]. In order to check the sole role of Na^+/Ca^{2+} exchange in the next (4th) series of experiments, the variation of hydration and $^{45}Ca^{2+}$ uptake in tissues are investigated by incubation of tissue samples in 100% Na and 50% Na PS. As can be seen in **Figure 4**, the incubation of tissue samples in 50% Na PS with $^{45}Ca^{2+}$ leads to the dehydration in ET of young and old animals (**Figure 4(a)**). The same effect is observed in liver tissue of young animals (**Figure 4(b)**) and also in the spleen tissue of young and old ones (**Figure 4(c)**). In liver tissue of old animals there is weak increase of hydration (**Figure 4(b)**). There must be checked the increase of $^{45}Ca^{2+}$ uptake in ET and its high level in ET of old animals than that in ET of young ones (**Figure 4(d)**). The decrease of $^{45}Ca^{2+}$ uptake in 50% Na PS is observed in NET of young rats (**Figure 4(e)**,

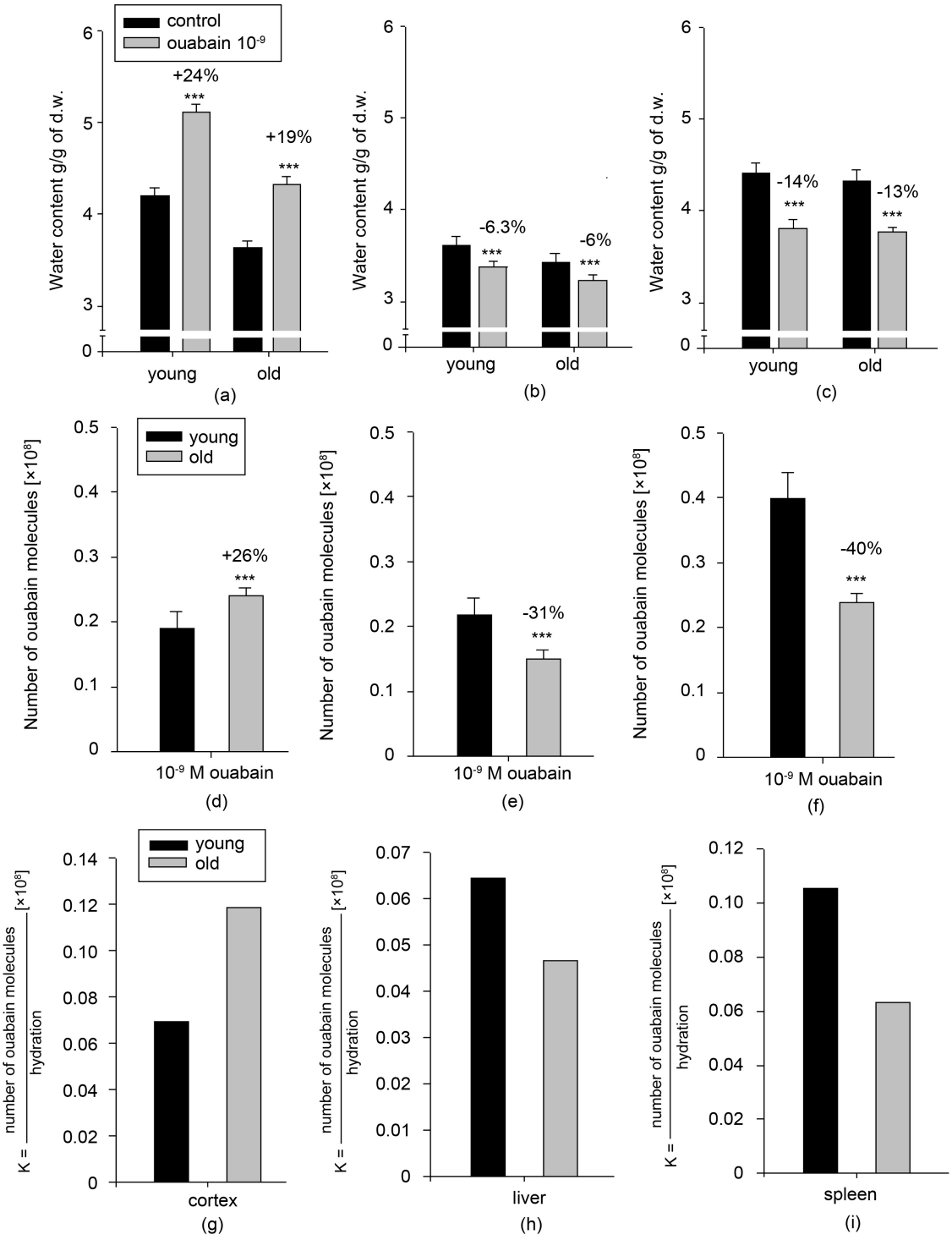

Figure 3. The effect of [^{3}H]-ouabain at 10^{-9}M on water content and number of ouabain molecules in brain cortex, liver and spleen tissues. Black bars on (a)-(c) indicate the mean value of water content in the tissues of control animal group (i/p injected by PS). Gray bars on (a)-(c) indicate the mean value of water content in the tissues of experimental animal group (i/p injected by [^{3}H]-ouabain at 10^{-9} M). Each bar represents the mean ± SEM (n = 45). (d)-(f) demonstrate the number of ouabain molecules in young (black bars) and old (gray bars) animals' tissues. The numbers in % indicate the difference between levels of hydration and the number of ouabain molecules. (g)-(i) indicate the ratio between the number of ouabain molecules and the level of water content. The symbol (***) indicates $p < 0.005$. All data were obtained from three independent experiments.

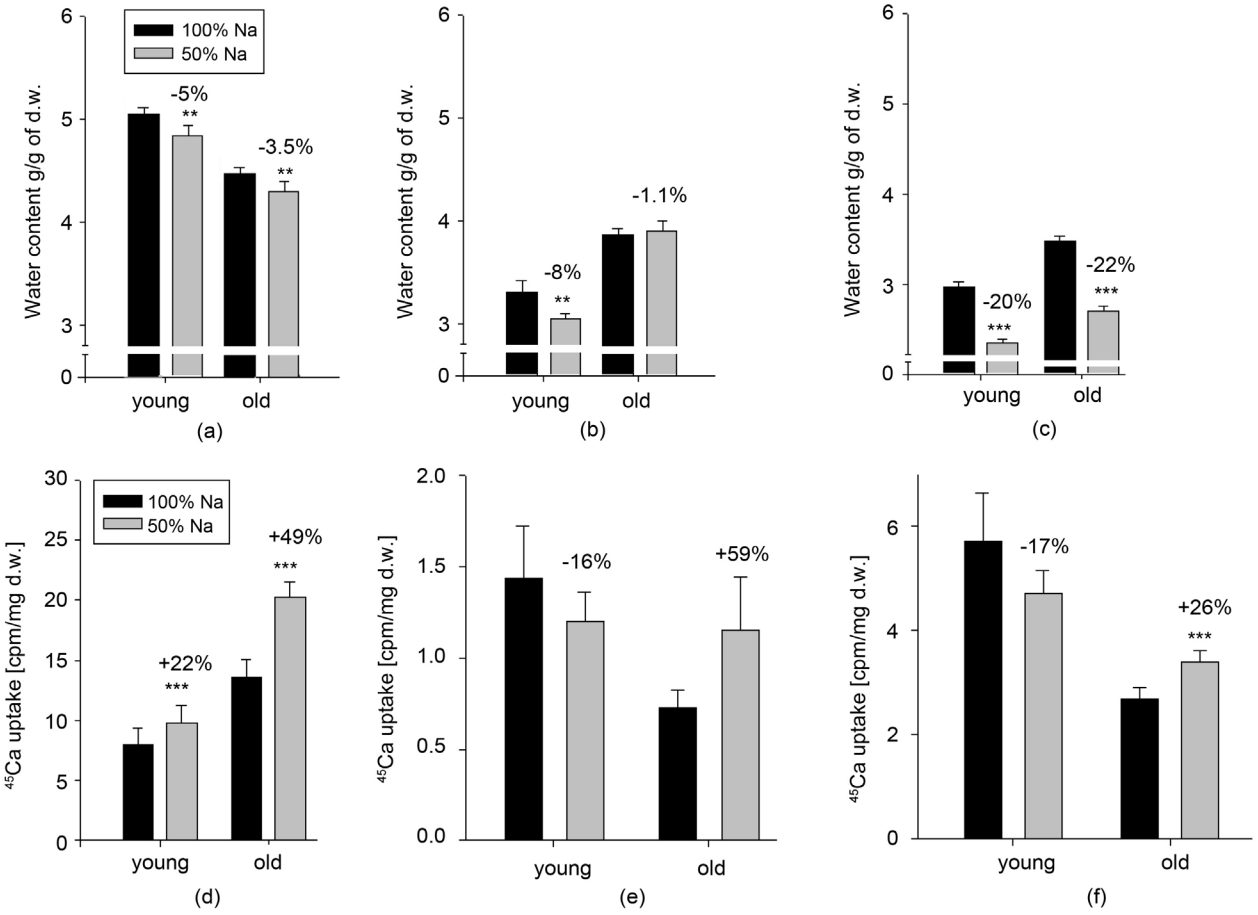

Figure 4. The comparative study of water content (a)-(c) and $^{45}Ca^{2+}$ uptake (d)-(f) in brain cortex, liver and spleen tissues samples incubated in 100% Na PS (black bars) and in 50% Na PS (gray bars). Each bar represents the mean ± SEM (n = 45). The symbols (**) and (***) indicate $p < 0.01$ and $p < 0.005$, respectively. The numbers in % indicate the difference between levels of hydration and $^{45}Ca^{2+}$. All data were obtained from three independent experiments.

Figure 4(f)) and the opposite effect (expressed increase of $^{45}Ca^{2+}$ uptake in 50% Na PS) is received in the same tissues of old animals (Figure 4(e), Figure 4(f)).

To estimate the role of Na^+/K^+ pump in the next (5^{th}) series in incubated solutions, besides $^{45}Ca^{2+}$, there is added non radioactive ouabain at 10^{-4}M. In such case the water content in all investigated tissues (after their incubation in 50% Na PS) is decreased (Figure 5(a), Figure 5(c)). The $^{45}Ca^{2+}$ uptake in ET of young rats is slowly decreased but it is expressively increased in ET of old ones (Figure 5(d)). In liver tissue of young and old rats $^{45}Ca^{2+}$ uptake is decreased (Figure 5(e)), meanwhile in spleen tissue (Figure 5(f)) it has smaller increase in tissues of young animals and expressively higher increase in tissues of old ones.

In the next group of animals (6^{th} series) the effect of nM non-radioactive ouabain is studied. In such conditions the water content in all investigated tissues incubated in 50% Na PS obviously decreases (Figure 6(a), Figure 6(c)) and is very expressive both in young and old rats' spleen tissues (Figure 6(c)). The $^{45}Ca^{2+}$ uptake in brain cortex tissue of young and old rats is expressively increased in 50% Na PS. The identical data have been received in liver tissue of old

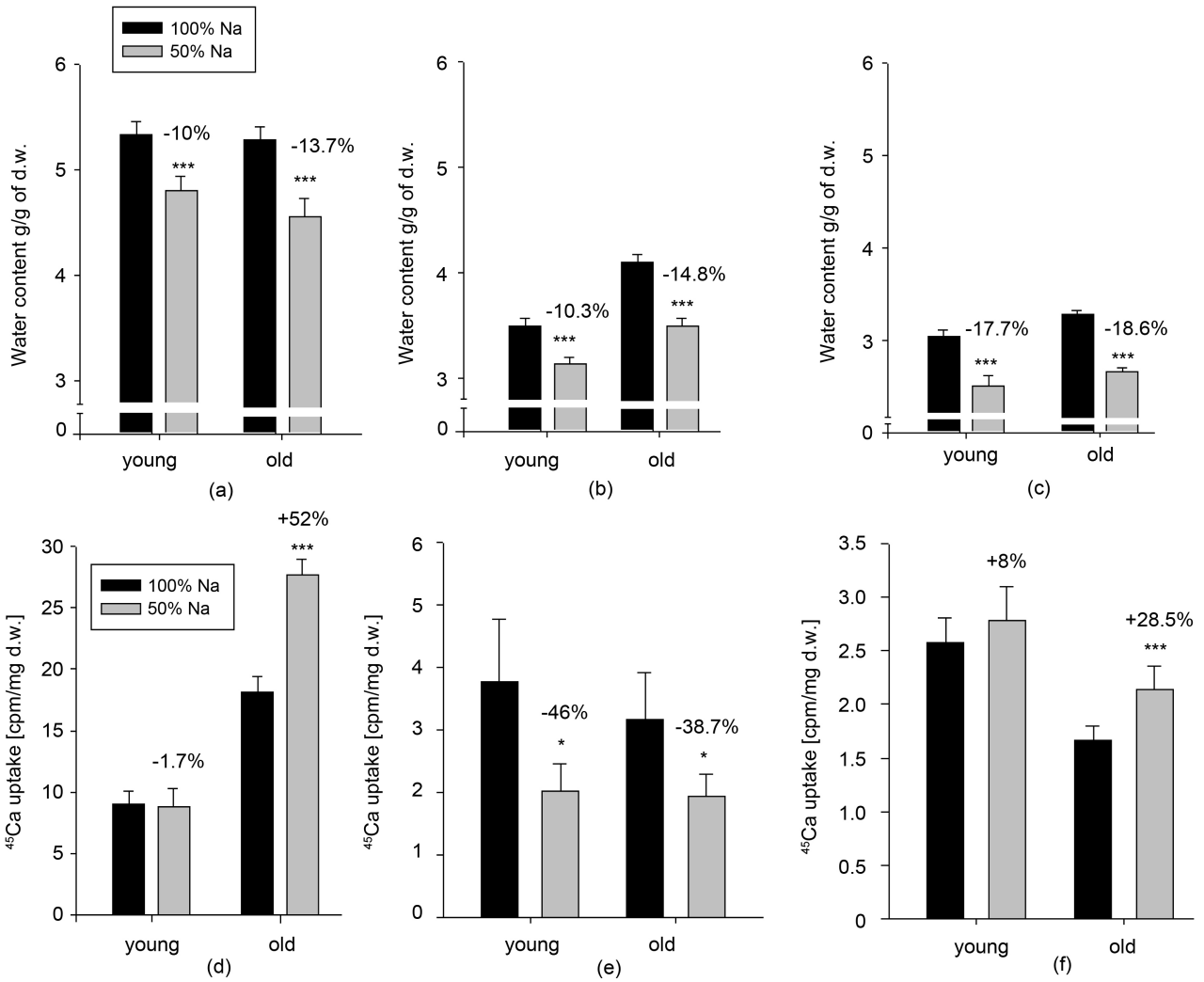

Figure 5. The variation of water content and $^{45}Ca^{2+}$ uptake in tissue samples incubated in 100% Na PS (black bars) and 50% Na PS (gray bars) in addition to non radioactive ouabain at 10^{-4} M ouabain. (a)-(c) the data of water content in brain cortex (a), liver (b) and spleen (c) tissues' samples. (d)-(f) data of $^{45}Ca^{2+}$ uptake in brain cortex (d), liver (e) and spleen (f) tissues. Each bar represents the mean ± SEM (n = 45). The symbols (*) and (***) indicate $p < 0.05$ and $p < 0.005$, respectively. The numbers in % indicate the difference between levels of hydration and $^{45}Ca^{2+}$. All data were obtained from three independent experiments.

animals (Figure 6(e)). The variation of $^{45}Ca^{2+}$ uptake in liver tissue of young animals is not so different in comparison with that in 100% Na PS (Figure 6(e)). The $^{45}Ca^{2+}$ uptake in spleen tissue of young rats in 50% Na PS is higher than that in 100% Na PS (Figure 6(f)). As for the $^{45}Ca^{2+}$ uptake, in spleen tissue of old animals it is lower in 50% Na PS than that in 100% Na PS (Figure 6(f)).

Previously it has been shown that cell swelling leads to the stimulation of cell metabolism by the increase of membrane functionally active protein molecules [12].

To evaluate the metabolic dependency of ET and NET hydration and their age dependency in the next (7^{th}) series of experiments the tissue hydration of animals drinking DW for 10 days is studied. After this the rats are i/p injected by PS and the results of these experiments are compared with that received in control

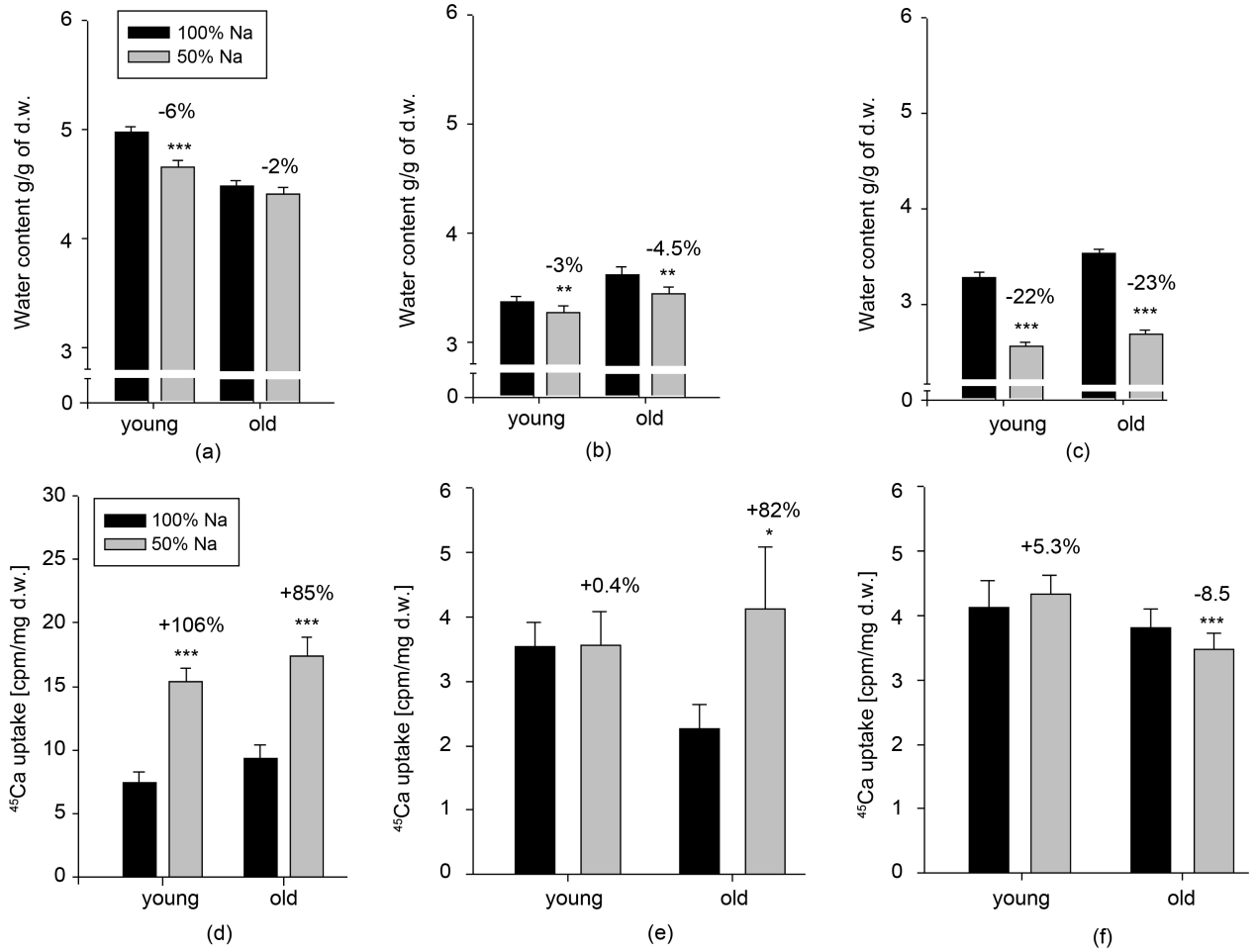

Figure 6. The variation of water content and $^{45}Ca^{2+}$ uptake in tissue samples incubated in 100% Na PS (black bars) and 50% Na PS (gray bars) in addition to non radioactive ouabain at 10^{-9} M ouabain. (a)-(c) the data of water content in brain cortex (a), liver (b) and spleen (c) tissue samples. (d)-(f) data of $^{45}Ca^{2+}$ uptake in brain cortex (d), liver (e) and spleen (f) tissues. Each bar represents the mean ± SEM (n = 45). The symbols (**) and (***) indicate $p < 0.01$ and $p < 0.005$, respectively. The numbers in % indicate the difference between levels of hydration and $^{45}Ca^{2+}$ uptake. All data were obtained from three independent experiments.

groups of animals drinking the tap water and also being injected by PS (**Figure 1**, black bars). In **Figure 7** can be seen that in such experimental conditions the hydration process in ET and NET is also different. In ET the over-hydration is observed both in young as well as in old animals (**Figure 7(a)**), but in NET the opposite effect (dehydration) is demonstrated (**Figure 7(b)**, **Figure 7(c)**).

To evaluate the role of Na^+/K^+ pump on tissue hydration and ouabain binding in the next series the new animal groups are taken. Each (young and old) group of animals drinks DW (as it was shown above) and then is i/p injected by [^{3}H]-ouabain at 10^{-4}M. Data of hydration and ouabain binding are compared with that received in similar experiments where the animals drink tap water and are also i/p injected by [^{3}H]-ouabain at 10^{-4}M (**Figure 2**). The results of this comparative data are different. As can be seen in **Figure 8** in the brain cortex tissue of young animals (drinking DW) over-hydration is observed, while in the similar group of old animals the opposite effect (dehydration) is demonstrated

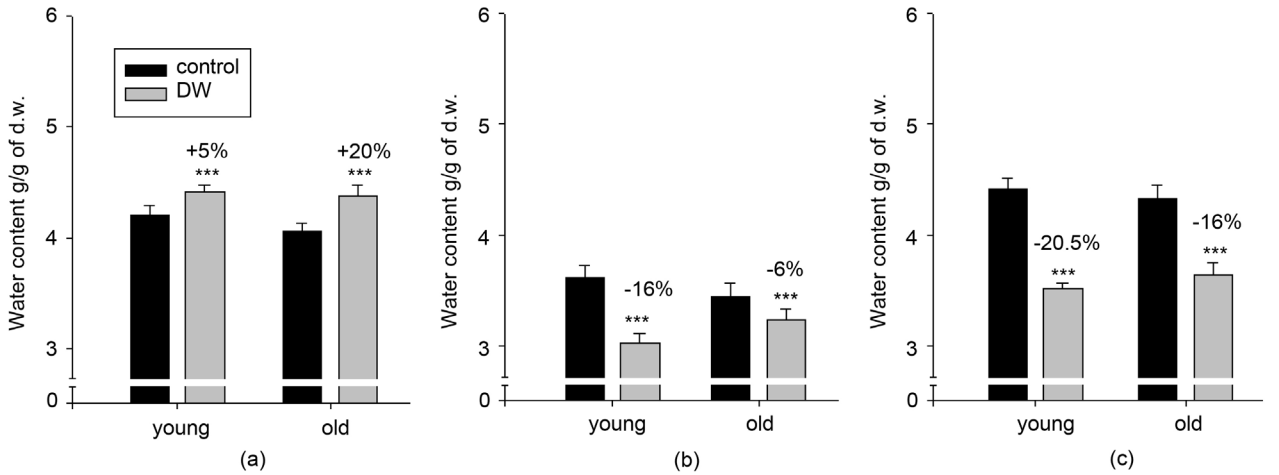

Figure 7. The variation of water content in brain cortex (a), liver (b) and spleen (c) tissues. Black bars indicate the mean value of water content in the tissues of control animal group drinking the tap water and being i/p injected with PS. Gray bars indicate the mean value of water content in the tissues of experimental animal group drinking DW and being i/p injected with PS. Each bar represents the mean ± SEM (n = 45). The symbol (***) indicates $p < 0.005$. The numbers in % indicate the difference between levels of hydration. All data were obtained from three independent experiments.

(Figure 8(a)). In liver tissue of young animals the small dehydration and in old rats an over-hydration are revealed (Figure 8(b)). In spleen tissues of both animal groups weak over-hydration is observed (Figure 8(c)). As for the ouabain binding, it must be noted that in brain cortex and liver tissues of young and old rats (drinking DW) the number of ouabain molecules in cell membrane has significantly been decreased (Figure 8(d), Figure 8(e)) and is expressively low in brain cortex tissue (Figure 8(d)) compared with that of animals drinking tap water. In spleen tissue of young rats the number of ouabain molecules increases but in old ones it decreases (Figure 8(f)). The coefficients of relationship of number of ouabain molecules and the level of hydration in brain cortex of young and old animals are nearly equal to each other (Figure 8(g)). In liver tissue of young rats it is lower compared with that in old ones (Figure 8(h)) and in spleen tissue (Figure 8(i)) this coefficient in young rats is higher than that in old ones. Another data have been received when the identical experiments have been carried out in animals drinking DW but being i/p injected by [^{3}H]-ouabain at 10^{-9}M. The data of these experiments have been compared with similar data where animals drink tap water and are injected by [^{3}H]-ouabain at 10^{-9}M. (Figure 9(a), Figure 9(c)) shows that in ET as well as in NET significant dehydration is observed. Besides, there is a sharp correlation between hydration and binding of ouabain molecules in all investigated tissues (Figure 9(d), Figure 9(f)). It must be noted that the decrease of number of ouabain molecules in young animals' liver and spleen tissues was more pronounced than that in old ones (Figure 9(e), Figure 9(f)). The ratio of the number of ouabain molecules to the level of water content is most pronounced in tissues of brain cortex and liver of old animals (Figure 9(g), Figure 9(h)) and, on the contrary, in the spleen tissue of young rats this ratio is higher (Figure 9(i)) than that in the old ones.

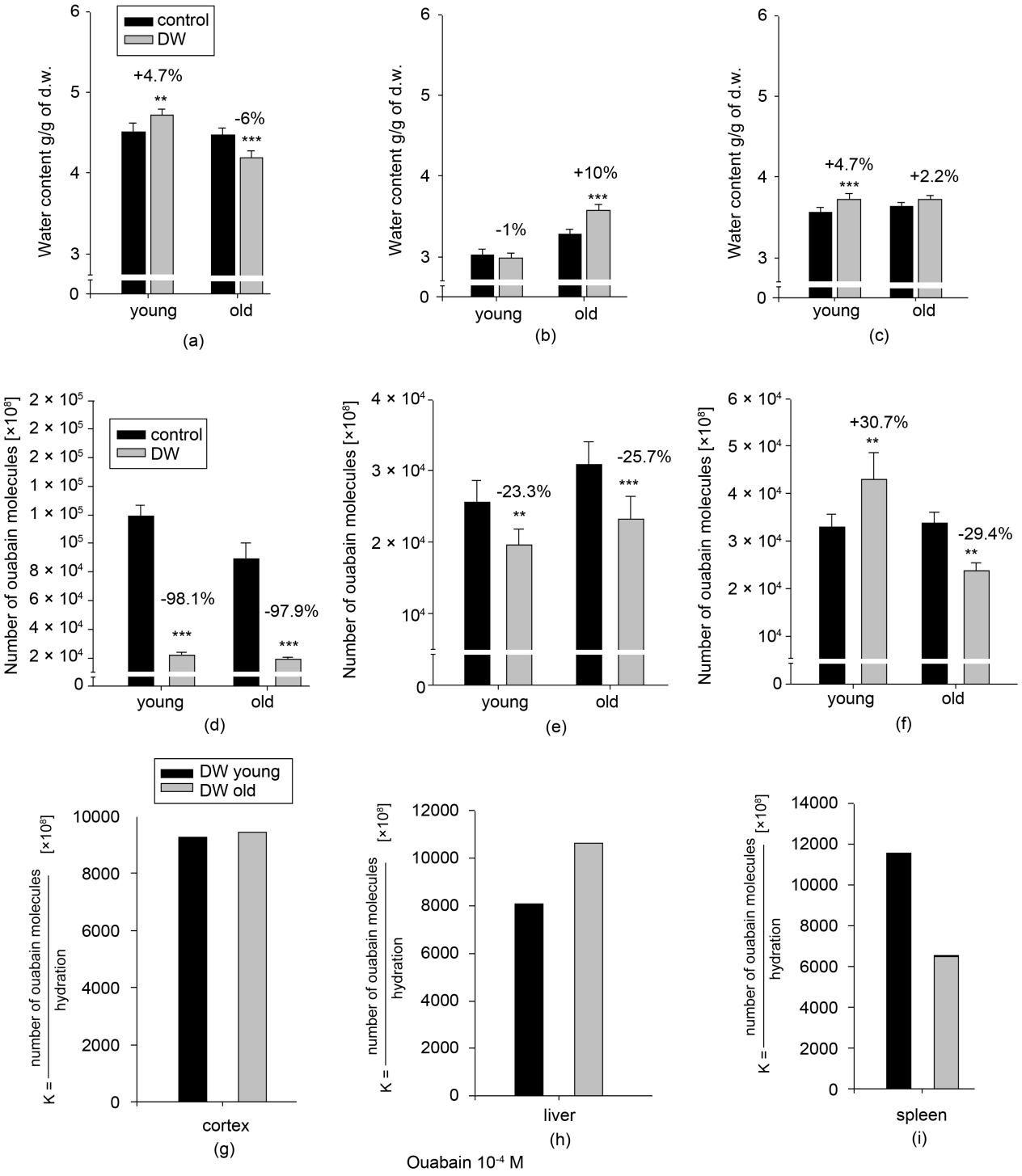

Figure 8. The effect of 10^{-4}M ouabain on hydration and ouabain binding in brain cortex, liver and spleen tissues. Black bars on (a)-(c) indicate the mean value of water content in the tissues of control animal group (i/p injected with [^{3}H]-ouabain at 10^{-4}M). Gray bars on (a)-(c) indicate the mean value of water content in the tissues of experimental animal group drinking DW and after 10 days being i/p injected with [^{3}H]-ouabain at 10^{-4}M. (d)-(f) demonstrate the number of ouabain receptors in tissues of young (black bars) and old (gray bars) animals. Each bar represents the mean ± SEM (n = 45). The symbols (**) and (***) indicate $p < 0.01$ and $p < 0.005$, respectively. The numbers in % indicate the difference between levels of hydration and the number of ouabain molecules. (g)-(i) indicate the ratio between the numbers of ouabain molecules and the level of water content. All data were obtained from three independent experiments.

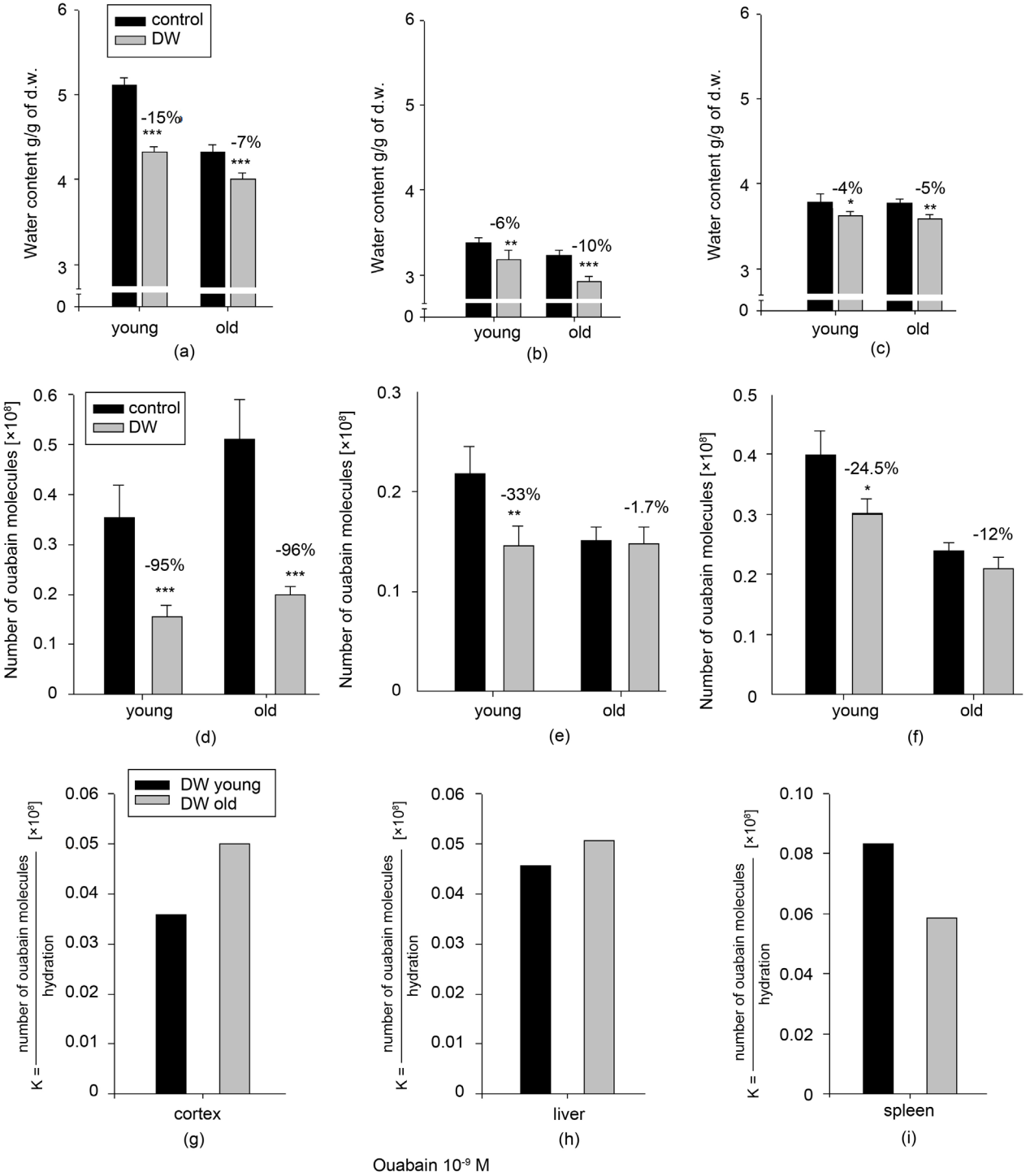

Figure 9. The effect of 10^{-9} M ouabain on hydration and ouabain binding in brain cortex, liver and spleen tissues. Black bars on (a)-(c) indicate the mean value of water content in the tissues of control animal group (i/p injected with [^{3}H]-ouabain at 10^{-9}M). Gray bars on (a)-(c) indicate the mean value of water content in the tissues of experimental animal group drinking DW and after 10 days being i/p injected with [^{3}H]-ouabain at 10^{-9}M. (d)-(f) demonstrate the number of ouabain receptors in tissues of young (black bars) and old (gray bars) animals. Each bar represents the mean ± SEM (n = 45). The symbols (*), (**) and (***) indicate $p < 0.05$, $p < 0.01$ and $p < 0.005$, respectively. The numbers in % indicate the difference between levels of hydration and the number of ouabain molecules. (g)-(i) indicate the ratio between the number of ouabain molecules and the level of water content. All data were obtained from three independent experiments.

4. Discussion

The obtained data that the cooling leads to dehydration in ET and hydration in NET (**Figure 1**) can be explained by low and high permeability of cell membrane for water in ET and NET, respectively. In neuronal tissue (ET) the ionic transporting systems (such as Na^+/K^+ pump and Na^+/Ca^{2+} exchange) are able to generate the osmotic gradients on the cell membrane, while NET cells are unable to generate these gradients on their membranes because of high proton (H^+) permeability [5]. Therefore, the generation of water efflux in ET cells takes place due to the activation of electrogenic ion transporting mechanisms as well as by endogen water formation in process of intracellular oxidation, while in NET cells the water efflux generates only by the formation of the endogen water. From this point of view, it is clear that there are different effects of cooling on ET and NET hydrations. Cooling dehydration effect in ET can be explained by the reciprocal correlation between Na^+/K^+ pump and RNa^+/Ca^{2+} exchange [14]. The cooling induced depression of electrogenic Na^+/K^+ pump activity leads to the activation of RNa^+/Ca^{2+} exchange, which is less sensitive to temperature because of its diffusion nature. Thus, ET dehydration by cooling can be explained by the activation of RNa^+/Ca^{2+} exchange leading to $[Ca]_i$-induced sol-gel transition of cytoplasm by formation of actin networks mediated by actin binding proteins of intracellular filaments and cytoskeleton [15]. The cooling-induced hydration of NET can be a result of depression of intracellular oxidative processes and production of endogen water. The latter has induced the water efflux from the cells, which besides balancing the water uptake has in hibitory effect on diffusion of osmolytes by cells (**Figure 1(b)**, **Figure 1(c)**).

It is well established that Na^+/K^+-ATPase in ET cells has three catalytic isoforms with different affinity to ouabain (inhibitor for Na^+/K^+-ATPase), while in NET cells only the low affinity isoforms (α_1) are expressed [16]. The obtained data indicate that 10^{-4} M ouabain (agonist for α_1 isoform) induces Na^+/K^+ pump inactivation and also leads to the opposite effect on ET and NET hydrations: hydration in the brain cortex and dehydration in the liver and spleen tissues (**Figure 2**). At first sight reverse effects of cooling and [^{3}H]-ouabain at 10^{-4}M on hydration of tissues are controversial to the abovementioned explanation of cooling effect. The Na^+/K^+ pump inhibition on the one side leads to hydration and on the other side to dehydration effect by activation of the RNa^+/Ca^{2+} exchange. In tissues of old animals the initial $[Ca]_i$ level is higher [17] than that in the same tissues of young ones where the rate of RNa^+/Ca^{2+} exchange is low. Therefore, in old animals the brain cortex hydration induced by Na/K pump is more pronounced than that in young animals' brain cortex tissue (**Figure 2(a)**). The dehydration effect in NET induced by Na^+/K^+ pump inhibition can be explained by the decrease of intracellular pH, which brings to the depression of water extrusion from mitochondria [18].

In spite of the fact that [^{3}H]-ouabain at 10^{-4}M in old animals' brain cortex leads to more pronounced hydration effect than that in young ones, the ouabain

binding with cell membrane is decreased in old animals (**Figure 2(d)**). In earlier experiments can be observed that there is a positive correlation between membrane surface and the number of ouabain binding sides in the membrane [12], while the affinity of these receptors to ouabain decreases by the increase of $[Ca]_i$ [19]. Therefore, the decrease of ouabain binding in the brain cortex of old animals compared with that of young ones can be explained by high $[Ca]_i$ in old animals' tissue [17]. It is remarkable that there is an age-dependent increase of ouabain receptors' expression in NET which is more pronounced in liver (**Figure 2(e)**) than in spleen tissue (**Figure 2(f)**).

It is known that the effect of $[^3H]$-ouabain at 10^{-9}M (compared with $[^3H]$-ouabain at 10^{-4}M) has no different effect on Na^+/K^+ pump activity and brings to the activation of RNa^+/Ca^{2+} exchange accompanied by the increase of cAMP in neurons [20]. As the experiments have showed $[^3H]$-ouabain at 10^{-9}M leads to hydration effect on brain cortex and dehydration on liver and spleen cells (**Figure 3(a)**, **Figure 3(c)**). It must be noted that at this ouabain concentration in brain cortex tissue of old animals the age-dependent increase of α_3 isoforms have been observed (**Figure 3(d)**), which is opposite to the effect of $[^3H]$-ouabain at 10^{-4}M (**Figure 2(d)**). In NET there is an age-dependent dehydration effect, which can be explained by cAMP-dependent activation of Ca-pump in the membrane of endoplasmic reticulum (ER), which through ER-mitochondria junction stimulates the generation of water efflux from mitochondria, which leads to transition from sole into gale state [15]. The data of ouabain binding decrease in aging in NET can be explained by accumulation of $[Ca]_i$ because of weakening of cAMP-dependent Ca pump in ER membrane (**Figure 3(e)**, **Figure 3(f)**).

The results in *in vitro* experiments, where the slices of ET and NET have been incubated in 100% and 50% NaCI containing PS with $^{45}Ca^{2+}$, and their metabolic state is depressed, the similar effects have been demonstrated in the brain cortex tissue hydration as in case of cooling (**Figure 1(a)**), the brain cortex tissue is dehydrated (**Figure 4(a)**). In NET, with the exception of liver tissue of old rats, the pronounced dehydration effect has been observed (**Figure 4(b)**, **Figure 4(c)**).

The age-dependent weakening of ET hydration (**Figure 4(a)**) when the concentration of $[Na]_o$ decreased, is accompanied with the activation of the RNa^+/Ca^{2+} exchange. This tissue dehydration brings the $^{45}Ca^{2+}$ uptake to increase in young as well as in old animals (**Figure 4(d)**), but in old animals the level of $^{45}Ca^{2+}$ uptake is higher than that in young ones (because of high content of $[Ca]_i$ in their brain cortex tissue). However, such correlation between the $^{45}Ca^{2+}$ uptake and tissue hydration in liver and spleen tissues are absent (**Figure 4(e)**, **Figure 4(f)**). In NET the decrease of $[Na]_o$ leads to dehydration in young animals, which accompanied by decrease of $^{45}Ca^{2+}$ uptake by cells, while in old animals' liver tissue there is non-significant increase of the $^{45}Ca^{2+}$ uptake (**Figure 4(e)**). Although in the spleen tissue the pronounced dehydration effect has been

observed (**Figure 4(c)**), the results of the $^{45}Ca^{2+}$ uptake are similar to that in liver tissue (**Figure 4(f)**). The data that the initial the $^{45}Ca^{2+}$ uptake by brain cortex tissue is higher than that in NET and the absence of activation effect of decreased $[Na]_o$ on the $^{45}Ca^{2+}$ uptake in young animals allow us to suggest on the non essential direct effect of RNa^+/Ca^{2+} exchange in metabolic regulation of cell hydration in NET. The non significant effect of decreased $[Na]_o$ on NET hydration, which accompanied by the increase of $^{45}Ca^{2+}$ uptake in old animals, probably can by explained by the activation effect of calmodulin on Na^+/Ca^{2+} exchange in the forward mode, which leads to replace the intracellular $^{40}Ca^{2+}$ by $^{45}Ca^{2+}$. This suggestion cannot be final and needs to have a special investigation.

The data of $[^3H]$-ouabain (at $10^{-4}M$) and $^{45}Ca^{2+}$ effects in 50% Na PS, where the role of pump is excluded, demonstrates the dehydration in all tissues (**Figure 5(a)**, **Figure 5(c)**). As it has been predicted, the decrease of $[Na]_o$ when Na^+/K^+ pump is in inactive state, the dehydration and $^{45}Ca^{2+}$ uptake in brain cortex is more pronounced (**Figure 5(a)**, **Figure 5(d)**) than when the pump is active (**Figure 4(a)**, **Figure 4(d)**). In liver tissues the age-dependent weakening dehydration effect is accompanied by depression of $^{45}Ca^{2+}$ uptake (**Figure 5(b)**, **Figure 5(e)**) and in spleen tissue there is an age-dependent strengthening effect (**Figure 5(c)**, **Figure 5(f)**). From these data can be suggested that Na^+/K^+ pump inactivation leads to the activation of Na^+/H^+ exchange in the reverse mode, which brings to inhibition of RNa^+/Ca^{2+} exchange and activation of mitochondrial function leading to tissue dehydration.

The data that 10^{-9} M ouabain in 50% Na PS has effects in ET can be explained by age-dependent weakening RNa^+/Ca^{2+} exchange (**Figure 6(a)**) led to age-dependent decrease of $^{45}Ca^{2+}$ uptake (**Figure 6(d)**). However, in liver tissue the age-dependent weakening dehydration effect leads to the age-dependent increase of $^{45}Ca^{2+}$ uptake (**Figure 6(e)**) but in spleen it is accompanied by age-dependent decreasing effect of Ca^{2+} uptake (**Figure 6(f)**). Since the Na gradient on the membrane serves as the energy source for both, Na^+/H^+ exchange and RNa^+/Ca^{2+} exchange, the effect of $[Na]_o$ on tissue hydration and $^{45}Ca^{2+}$ uptake can be explained by competitive interaction between Na^+/H^+ and RNa^+/Ca^{2+} differences between age-dependent Ca uptake in liver and spleen tissues probably can be explained by different expressions of these exchangers, which could serve as a subject for special investigation. The different age-dependent ion transporting mechanisms between these tissues indicate the age-dependent increase of Na^+/K^+ pump units in liver while it is absent in the spleen (**Figure 2**). The cell hydration leads to the activation of cell metabolism [2] [12]. Therefore, it has been suggested that by supplying animals DW instead of regular tap water, it could stimulate the metabolic activity of tissues. The data obtained on animals drinking during 10 days DW instead of tap water, indicates that in ET there is an age-dependent increase of tissue hydration (**Figure 7(a)**), while in NET an age-dependent dehydration is observed (**Figure 7(b)**, **Figure 7(c)**), *i.e.* there is the opposite picture to that in case of cooling (**Figure 1(a)**, **Figure 1(c)**). From these data can be

concluded that DW-induced activation of mitochondrial activity leads to the water efflux from the cells bringing to dehydration (transition from sole into gel state) in NET. This explanation supports the data that old animals' DW-induced dehydration effect in NET is less than that in young ones (**Figure 7(b)**, **Figure 7(c)**).

The data on Na^+/K^+ pump inactivation by 10^{-4}M ouabain indicate that in ET of young and old animals there are hydration and dehydration effects, respectively (**Figure 8(a)**). It is worth to note that the number of ouabain binding has pronounced decrease in the membrane in case of animals drinking DW compared with those drinking tap water (**Figure 8(d)**). These data indicate on the increase of expression of Na^+/K^+ pump units in ET of old animals (**Figure 7(d)**, **Figure 7(g)**). Probably, the increase of pump units in ET of old animals is the compensated response of brain tissue for weakening pump function because of high $[Ca]_i$. It seems extremely interesting, that Na^+/K^+ pump inactivation leads to non-significant effect on liver dehydration of young animals but has pronounced hydration effect in old ones (**Figure 8(b)**), while in spleen tissue of both age groups the hydration is observed (**Figure 8(c)**), which accompanied by the over-expression of pump units in aging animals, has more pronounced character in liver tissues (**Figure 8(h)**, **Figure 8(i)**). These data clearly indicate that the electrogenic ion transporting mechanisms such as Na^+/K^+ pump and Na^+/Ca^{2+} exchange and endogen water molecules' formation as a result of oxidative processes have the key role in regulation of neuronal hydration. Meanwhile, in NET because of their cell membrane higher permeability to water (H^+), the electrogenic properties of cell membrane are depressed and their hydration controlled by mitochondrial activity in its turn releases the production of water molecules in cytoplasm.

As it has been predicted, 10^{-9} M ouabain has dehydration effect on tissue, which is due to activation of RNa^+/Ca^{2+} exchange in ET (**Figure 9(a)**) and the activation of Na^+/H^+ exchange in NET (**Figure 9(b)**, **Figure 9(c)**). It is interesting that there is an age-dependent increase of α_3 receptors in the membrane (**Figure 9(g)**), which have RNa^+/Ca^{2+} function in neurons [21], while in NET tissues Na^+/H^+ exchange (**Figure 8(g)**). 10^{-9}M ouabain has an age-dependent dehydration effect on liver and spleen tissues, which is accompanied by the age-dependent increase of nM ouabain receptors in liver and its decrease in spleen membranes. Since the Na^+/K^+-ATPase isoforms in NET are absent, nM ouabain induced effect in NET can be explained by the activation of G protein in the membrane leading to the increase of the intracellular cAMP contents, which probably activates Na^+/H^+ exchange [22]. Therefore, it is suggested that in NET the nM ouabain brings to cAMP-dependent dehydration by stimulation of Na^+/H^+ exchange, while the brain tissue dehydration is the result of activation of cAMP-dependent RNa^+/Ca^{2+} exchange. Thus, the summary of the obtained data brings us to the conclusion that the over-expression of Na^+/Ca^{2+} exchanger in the brain cortex and the Na^+/K^+ pump units in the liver could be considered as

markers for aging.

5. Conclusions

1) Metabolic regulation of cell hydration in excitable tissue (brain cortex) is realized by the activation of electrogenic Na^+/K^+ pump and Na^+/Ca^{2+} exchange in the cell membrane and the formation of endogen water by mitochondrial activity.

2) Regulation of cell hydration in non-excitable tissue (liver, spleen) is carried out only by the activity of mitochondrial function.

3) Aging leads to an over-expression of Na^+/K^+ pump units in liver and Na^+/Ca^{2+} exchanger in brain cortex of rats.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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