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EFFECT OF SEX AND LOCALIZATION DEPENDENT DIFFERENCES OF NA,K-ATPASE PROPERTIES IN BRAIN OF RATS

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Na,K-ATPase is the main system effectively excluding the superfluous sodium out of the cells on the expense of energy derived from hydrolysis of ATP. In brain 3 different isoforms of the catalytic α-subunit are known. The present study was focused to energy utilization and ability to bind sodium by the Na,K-ATPase as well as expression of all 3 isoforms of the catalytic α-subunit concerning its sex specificity in two selected regions of the brain, in cortex and in cerebellum of rats. Western blot analysis showed higher expression of all 3 catalytic α-subunits of Na,K-ATPase in cerebellum when compared to cortex which was not followed by higher activity. On contrary the total activity of the enzyme was lower in cerebellum comparing with cortex in females with no significant localization dependent differences of activities in males. Concerning sex dependence only the expression of α3 isoform was higher in cortex of male rats with no differences in the levels of α1 and α2 isoforms. However, the total activity of Na,K-ATPase in cortex was similar in male and female groups. On the other hand in cerebellum of females the total activity of Na,K-ATPase was significantly lower as compared with males. The obtained data indicate localization and sex dependent variations in maintenance of sodium homeostasis in the brain.

Key words: *sodium pump, expression, enzyme kinetics, cerebellum, cerebral cortex, gender*

INTRODUCTION

In brain one of the main energy utilizers is the Na,K-ATPase consuming approximately 50% of its energy demand (1). This enzyme called also as sodium pump is responsible for maintenance of appropriate intracellular balance of sodium and potassium ions on the expense of energy derived from hydrolysis of ATP. In addition to its pump function, Na,K-ATPase serves as a signaling system and a cell adhesion molecule (2). The enzyme consists of two main subunits, the catalytic α-subunit and the β-subunit which serves as a chaperone, stabilizing the correct folding of the α-subunit to facilitate its delivery to the plasma membrane (3). In the cerebral tissue 3 isoforms of the catalytic α-subunit were identified (4-6). The isoform α1 is expressed ubiquitously in the brain tissue while the α2 isoform is expressed primarily in glial cells and developing neurons and α3 isoform is restricted to neurons (5, 7, 8). These isoforms differ in the ability to bind intracellular sodium as their K_{Na} value increases in the sequence α1< α2< α3. The α3 isoform has approximately threefold lower affinity to Na^+ compared with α1 (9-11). Beside the maintenance of intracellular homeostasis of ions the Na,K-ATPase seems to be involved also in other aspects of health condition of the brain (12) which is closely related to damage of blood brain barrier (13). It was documented that dysfunction of the Na,K-ATPase was accompanied with the rupture on blood-brain barrier (BBB) (14-17). It was shown that ouabain, a specific inhibitor of the Na,K-ATPase can produce alterations in the permeability of BBB as documented by increased passage of Evans Blue into cortical tissue (18). Experiments with water intoxication of rats showed higher susceptibility of female brains to this insult as the activity of Na,K-ATPase as well as the integrity of blood-brain barrier were more deteriorated as compared with male rats (19). Although several studies have demonstrated differential kinetic properties and protein expression of the various Na,K-ATPase α isoforms in the brain tissue of rodents, their activities and expression profile in both genders has not been thoroughly investigated yet. The present study was oriented to energy utilization and ability to bind sodium by the Na,K-ATPase as well as expression of all 3 isoforms of the catalytic α-subunit concerning its sex specificity in two selected regions of the brain, in cortex and in cerebellum in rats.

MATERIAL AND METHODS

Animal model

During the study, 12 weeks old Wistar rats of both genders (n = 9 in each group) were housed in groups of 3 in cages of the type T4 Velaz (Prague, Czech Republic) with bedding composed of wood shaving (exchanged daily). All rats were allowed free access to food and drinking water (*ad libitum*). The animal room was air-conditioned and the environment was continually monitored for the temperature of 23 ± 1°C with relative humidity of 55 ± 10%. Reaching the age of 16-weeks rats were sacrificed under thiopental anesthesia (50 mg/kg). Samples from cerebral cortex and cerebellum were immediately frozen in liquid

nitrogen and stored in –60°C for further investigations of Na,K-ATPase properties.

All procedures in this study were approved by the Institutional Animal Care Committee and their correspondence to IACUC was attested by State Veterinary and Food Administration of the Slovak Republic.

Assay of Na,K-ATPase activity

The plasmalemmal membrane fractions from whole cerebral cortexes and cerebellums were isolated according to (20). Amount of proteins was determined by the procedure as described previously (21) using bovine serum albumin as a standard.

All assays of the Na,K-ATPase activity were performed (22, 23) at 37°C using 10 μg·ml^{-1} of membrane protein in an assay buffer containing (in mmol·l^{-1}): 4 $MgCl_2$, 100 NaCl, 10 KCl and 50 TRIS (pH = 7.4). The samples were pre-incubated for 20 min in substrate-free medium. The enzyme reaction was initiated by addition of increasing amount of TRIS-ATP in the range of 0.16 – 8.00 mmol·l^{-1}. The reaction was stopped after 20 min by adding 12% ice-cold trichloracetic acid. The inorganic phosphorus generated from ATP hydrolysis was estimated according to (24). In order to establish the Na,K-ATPase activity, the ATP hydrolysis occurring in the presence of Mg^{2+} only, was subtracted. The enzyme kinetics for sodium activation was determined by the same way. The concentration of NaCl varied in the range of 2 – 100 mmol·l^{-1} and the amount of ATP was constant (8 mmol·l^{-1}). The kinetic parameters were evaluated by direct non-linear regression of the obtained data. Parameter V_{max} represent the theoretical maximal velocity of the enzyme reaction and K_m and K_{Na} represent the concentration of ATP or Na^+ necessary for half maximal activation of the enzyme.

Preparation of tissue fractions for electrophoresis and immunochemical Western blot analysis

The tissue samples from whole rat cerebral cortex and cerebellum were re-suspended in ice-cold buffer containing (in mmol·l^{-1}): 50 Tris-HCl, 250 sucrose, 1.0 dithiothreitol, 1.0 phenylmethylsulfonylfluoride (pH 7.4) and homogenized with a glass-teflon homogenizer. The homogenates were centrifuged at 800 × g for 5 min at 4°C, pellets after this centrifugation were discarded and the supernatants were centrifuged again at 16100 × g for 30 min. Following this second centrifugation the supernatants were discarded again and the pellets were re-suspended in homogenizing buffer supplemented with 0.2% Triton X-100 and centrifuged at 16100 × g for 5 min. The Triton X-100 soluble supernatants represented the particulate fractions. The protein concentrations were estimated according to (25). Samples of particular protein fractions (for α1-3 Na,K-ATPase subunits detection) containing equivalent amounts of proteins per lane (25 μg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis (SDS-PAGE). For Western blot assays separated proteins were transferred from gel to a nitrocellulose membrane overnight at 4°C. The quality of the transfer was controlled by Ponceau S staining of nitrocellulose membranes after the transfer. Specific antibodies against α1 (mouse monoclonal antibody from Sigma; product number A-277, RRID:AB_258030, in dilution 1:250, epitope is between amino acids 496 and 506 of the lamb kidney Na,K-ATPase α1 subunit), α2 (rabbit polyclonal antibody from Milipore; #07-674, RRID:AB_390164, in dilution 1:1000, synthetic peptide corresponding to amino acids 432-445 of human Na,K-ATPase α2, with an N-terminal cysteine added for conjugation purposes) and α3 (rabbit polyclonal antibody from Milipore; #06-172, RRID:AB_11213338, in dilution 1:1000, fusion protein derived the α3 subunit containing residues 320-514) subunits of Na,K-ATPase were used for the primary immunodetection. Peroxidase-labelled anti-mouse (from Cell Signaling; #7076, RRID:AB_330924, in dilution 1:1000) and anti-rabbit (from Cell Signaling; #7074S, RRID:AB_2099233, in dilution 1:1000) immunoglobulin were used as the secondary antibodies. Bound antibodies were detected by the enhanced chemiluminescence detection method (Amersham Imager 600). Densitometrical quantification of protein levels was performed by comparison to loading control β-actin (mouse monoclonal antibody (AC-15) from Abcam; ab6276, RRID:AB_2223210, in dilution 1:1000, epitope is on N-terminal of the beta isoform of actin) and corresponding anti-mouse secondary antibody and using an ImageJ program.

Statistical analysis

All investigated parameters are expressed as means ± SEM. ANOVA and Holm-Sidak test were used for statistical analysis. The differences were considered to be significant when the P-value was less than 0.05.

RESULTS

Enzyme kinetics

Studying the gender specificity of the Na,K-ATPase in cerebral cortex by activation of the enzyme with increasing concentrations of ATP we observed lower activities in the whole concentration range in males when comparing to females (*Fig. 1*). The difference increased stepwise with increasing concentrations of substrate from 8% observed in the presence of 0.16 mmol.l^{-1} of ATP to 18%, observed in the presence of 8 mmol.l^{-1}. Analysis of the data according to Michaelis-Menten equation resulted in significantly lower V_{max} value by 17% in the group of males without significant changes in the K_m value (*Fig. 2*). Activation of the Na,K-ATPase with increasing concentration of sodium showed similar activity in the whole concentration range in cortex of female and male rats (*Fig. 3*) resulting in similar V_{max} and K_{Na} values in both sex groups (*Fig. 4*).

In cerebellum of males we observed significantly higher activity of the Na,K-ATPase as compared with females for all applied concentrations of substrate. The effect represented 65% increase at 0.16 mmol.l^{-1} of ATP and at 8 mmol.l^{-1} the difference represented 100% (*Fig. 1*). Estimation of kinetic parameters resulted in significantly higher V_{max} by 77% and K_m by 30% values in cerebellum of males (*Fig. 2*).

Activation of the Na,K-ATPase from cerebellum with increasing concentration of sodium showed continual increase of its activity throughout the whole concentration range in males when comparing to females. The effect increased stepwise with increasing concentrations of Na^+ from 43% observed in the presence of 2 mmol.l^{-1} of NaCl to 74% observed in the presence of 100 mmol.l^{-1} (*Fig. 3*) resulting in significantly higher V_{max} value by 77% and K_{Na} value by 13% as compared to female group (*Fig. 4*).

Studying the influence of localization on the Na,K-ATPase in the brain of females we observed significantly lower activities in cerebellum as compared to cortex. Activation of the enzyme with increasing concentrations of ATP was followed by lower activity in cerebellum (*Fig. 1*) resulting in significant decrease of V_{max} value (by 60%) and also of K_m value by 22% (*Fig. 2*). When activating the enzyme with increasing concentration of sodium we observed similar effect (*Fig. 3*) resulting in lower value of V_{max} by (45 %) and lower value of K_{Na} by 15% (*Fig. 4*).

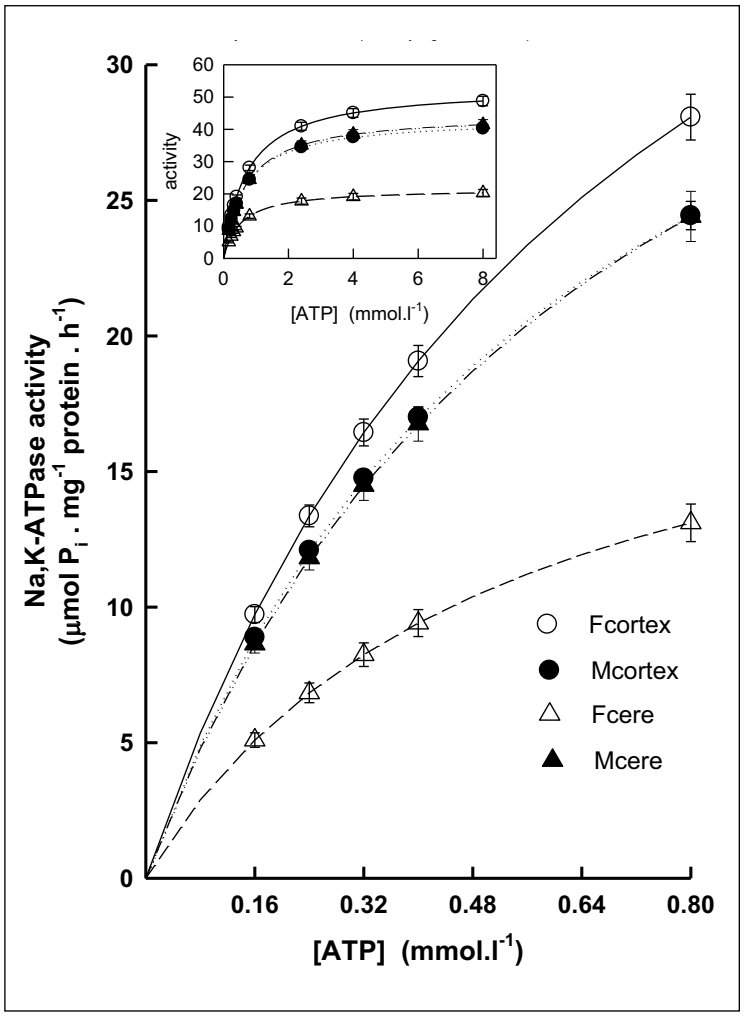

Fig. 1. Activation of the Na,K-ATPase by low concentrations of substrate ATP in female cerebral cortex (Fcortex), in female cerebellum (Fcere), male cerebral cortex (Mcortex) and in male cerebellum (Mcere). Inset: activation of the enzyme in the whole investigated concentration range of ATP.

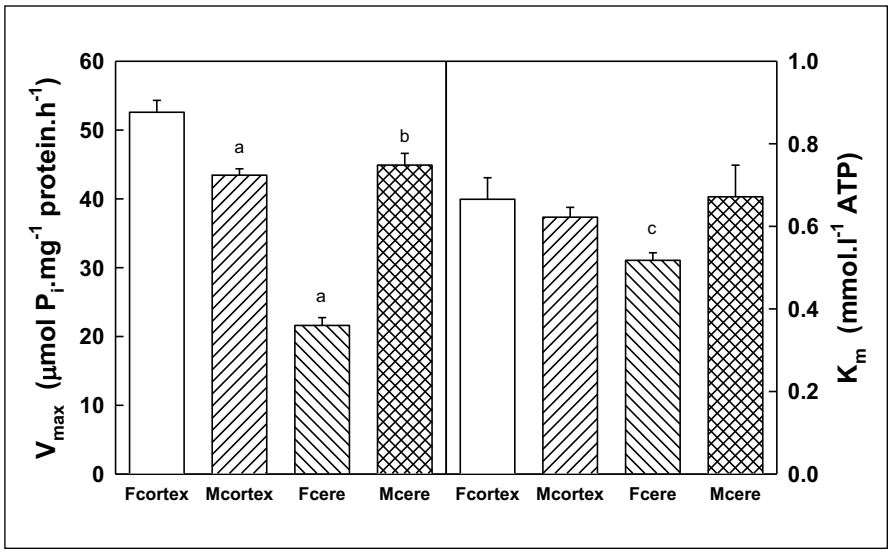

Fig. 2. Kinetic parameters of the Na,K-ATPase during activation with substrate ATP in female cerebral cortex (Fcortex), in female cerebellum (Fcere), male cerebral cortex (Mcortex) and in male cerebellum (Mcere). The parameter V_{max} represents the maximal velocity of enzyme reaction, K_m value refers to the concentration of ATP necessary for half maximal activation of the enzyme. Data represent mean ± SEM, n = 9 in each group. Significance a: $P < 0.001$ versus Fcortex, b: $P < 0.001$ versus Fcere, c: $P < 0.05$ versus Fcortex.

In the brains of males the activity of Na,K-ATPase was similar in cortex and in cerebellum throughout the applied concentration range of substrate ATP as well as cofactor sodium (*Figs. 1* and *3*). Evaluation of kinetic parameters resulted in similar values of V_{max}, K_m and K_{Na} in both investigated regions (*Figs. 2* and *4*).

Western blot analysis

Focusing on the sex differences of Na,K-ATPase expression, analysis of catalytic α-subunits by Western blot (*Fig. 5*) showed similar level of α1 subunit in cortex of female and male rats. In cerebellum of males the presence of α1 subunit was doubled as

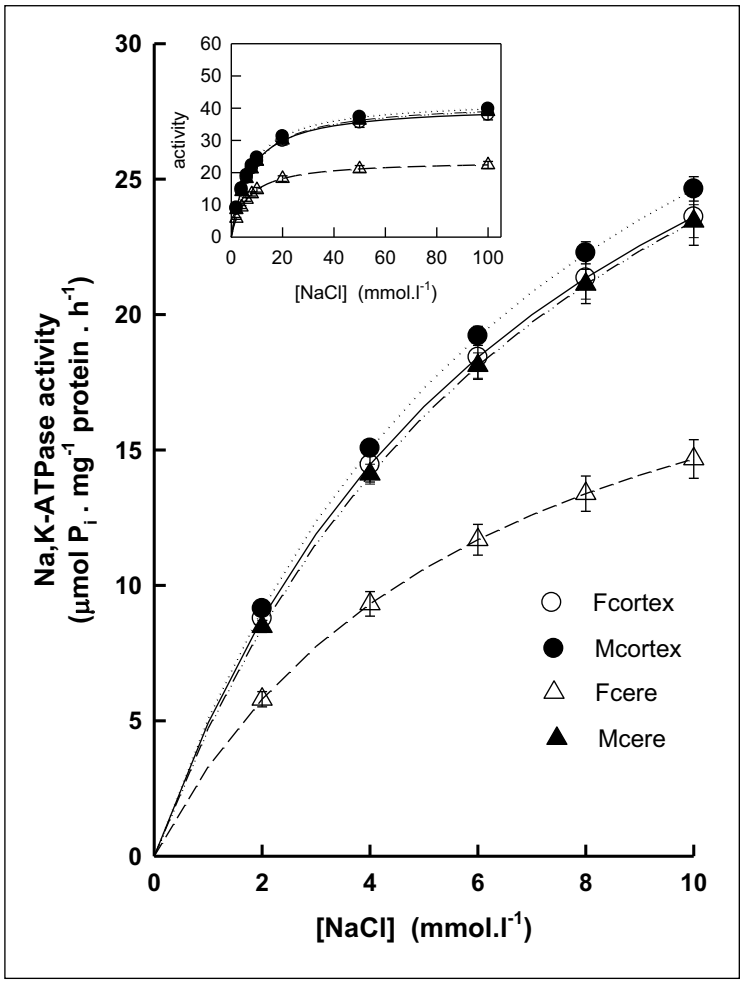

Fig. 3. Activation of the Na,K-ATPase by low concentrations of cofactor Na^+ in female cerebral cortex (Fcortex), in female cerebellum (Fcere), male cerebral cortex (Mcortex) and in male cerebellum (Mcere). Inset: activation of the enzyme in the whole investigated concentration range of NaCl.

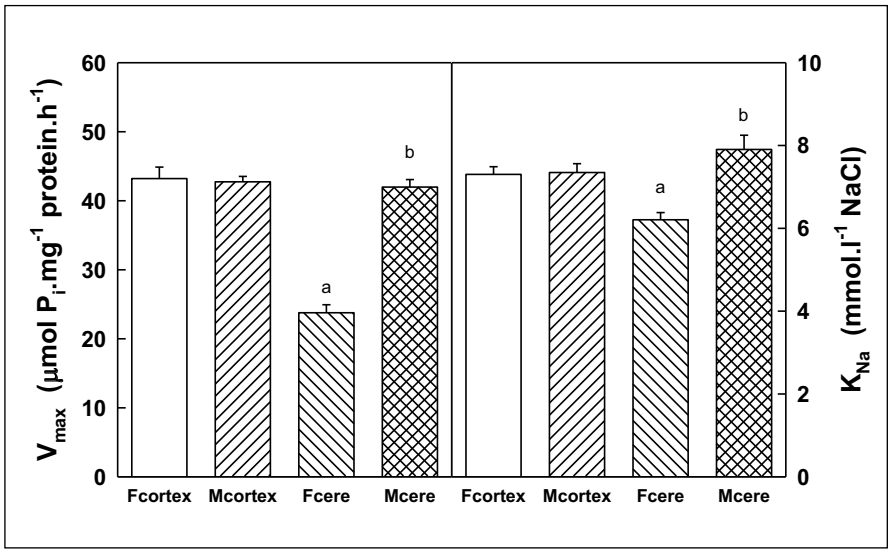

Fig. 4. Kinetic parameters of the Na,K-ATPase during activation with cofactor Na^+ in female cerebral cortex (Fcortex), in female cerebellum (Fcere), male cerebral cortex (Mcortex) and in male cerebellum (Mcere). The parameter V_{max} represents the maximal velocity of enzyme reaction, K_{Na} value refers to the concentration of Na^+ necessary for half maximal activation of the enzyme. Data represent mean ± SEM, n = 9 in each group. Significance a: $P < 0.001$ versus Fcortex, b: $P < 0.001$ versus Fcere.

compared with females. The presence of α2 in cortex as well as, in cerebellum was similar in males and females. The level of α3 subunit was significantly higher in cortex and also in cerebellum of males.

Focusing on differences of Na,K-ATPase levels in selected parts of rat brain the α1 subunit was more abundant in cerebellum as compared with cortex (*Fig. 5*). This elevation represented 70% in females and 215% in males. The level of α2 subunit was again higher in cerebellum by 550% in females and 500% as in males. The level of α3 subunit was also higher in cerebellum by 240% in females and 120% in males.

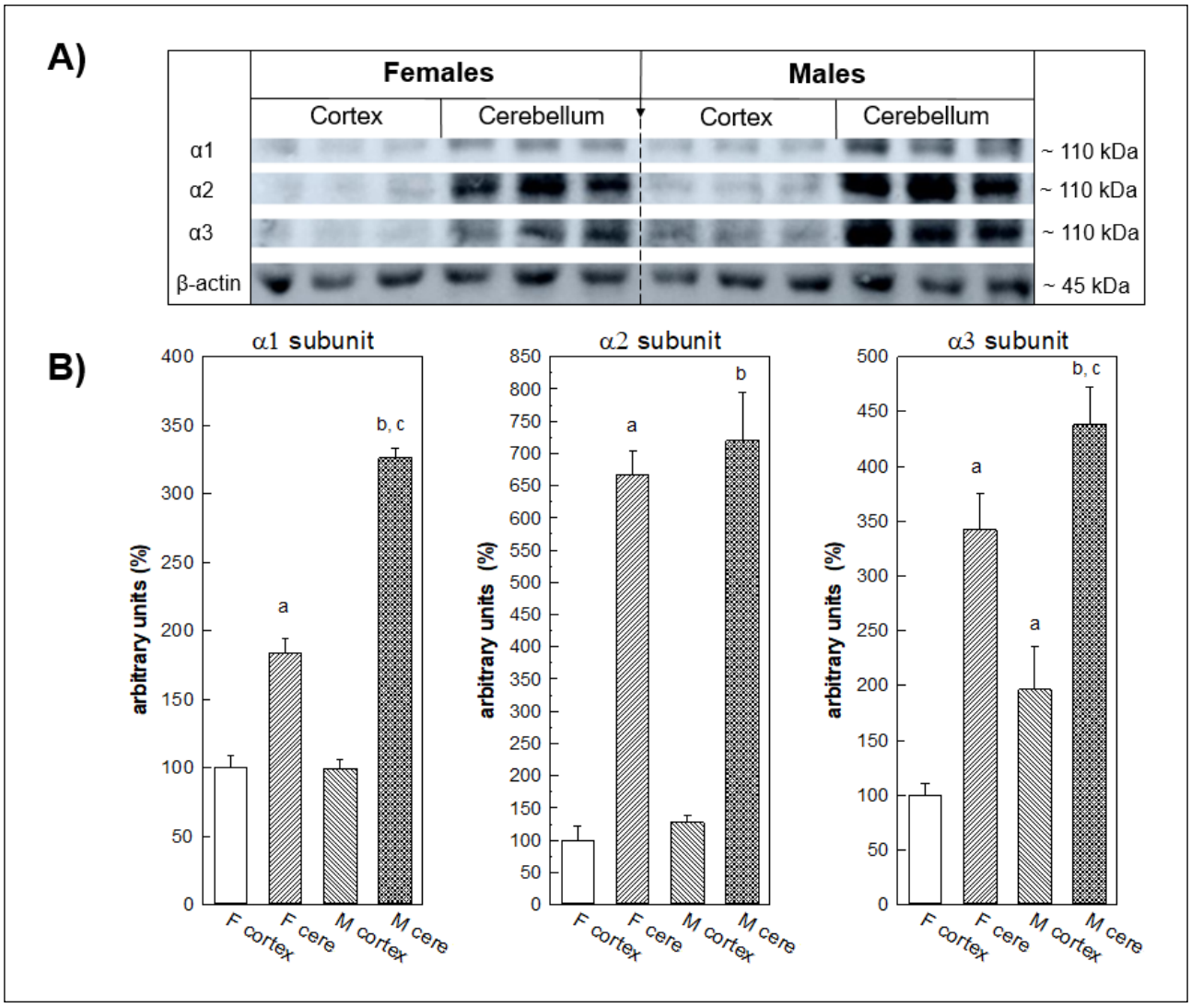

Fig. 5. A: Immunoblot analysis of α1-3 subunits of the Na,K-ATPase in female cerebral cortex (F cortex), in female cerebellum (F cere), male cerebral cortex (M cortex) and in male cerebellum (M cere). Representative blots of 3 samples from each group. B: Relative abundance of α1-3 subunits of the Na,K-ATPase. Relative densities of bands. Data represent mean ± SEM. Significance a: $P < 0.05$ versus F cortex, b: $P < 0.05$ versus M cortex, c: $P < 0.05$ versus F cere.

DISCUSSION

The disturbances in functioning of Na,K-ATPase in the brain are responsible for development of various brain-disorders *e.g.* hyponatremic encephalopathy (26), migraines (27), epilepsy (28), Alzheimer disease (29), *etc*. Based on the available literature it can be summarized that sex is an important variable influencing the predisposition to various pathophysiological events like encephalopathy (30) or altered synaptic plasticity (31). For example increased production of lipid peroxidation and nitric oxide were found with a higher amount in male mice than in female mice (32). Experimental inhibition of acetylcholine esterase showed that females were more susceptible than males with regard to brain Na,K-ATPase, indicating sexual dimorphism in treated rats (33). In our previous studies we have also documented sex dependent variations of the Na,K-ATPase in response to hyperglycemia in cortex and also in cerebellum (34, 35). Sex dependent variation in Na,K-ATPase properties of healthy subjects was also documented in forebrain (30, 36). In addition, localization dependent variation in the activity or the expression of Na,K-ATPase was documented in various parts of brain like cerebral cortex, hippocampus and brain stem (37-42). The localization dependent variations in the brain seem to be very important as it was emphasized also by altered expression of endocannabinoid-metabolizing enzymes in rats after treatment with antidepressant drugs (43). So in the present study we focused our attention to hypothetical sex and localization dependent variations in kinetic properties and expression of various isoforms of catalytic subunit of the Na,K-ATPase in the brains of healthy rats without any treatment.

Point of view: localization

Mapping the presence of all 3 catalytic α-isoforms of Na,K-ATPase in rat brains pointed to their higher expression in cerebellum than in cerebral cortex. This finding observed in males as well as in females may be ascribed to local synthesis of estrogen in cerebellum. Previous human and animal studies have evidenced that estradiol is synthesized locally within the cerebellum (44-49). The local synthesis of estradiol in cerebellum

in females and also in males seems to be a very important additional factor to the overall level of estradiol circulating in the blood. So the cerebellar Na,K-ATPase is probably subjected to estradiol more intensively than the enzyme in males. The involvement of estradiol in regulation of local protein synthesis (47, 50) may be responsible for higher presence of all 3 isoforms of catalytic α-subunit of Na,K-ATPase in cerebellum when compared to cerebral cortex. However, our studies of enzyme kinetics showed that the higher presence of α-subunit in cerebellum was not manifested in increased activity of the enzyme when compared to cortex. In females the number of active Na,K-ATPase molecules was significantly lower as indicated by lower V_{max} value in cerebellum when compared to cerebral cortex. This localization dependent decrease in activity seems to be partially compensated by improved binding properties of the enzyme for energy substrate ATP and also for sodium as indicated by lowered K_m and K_{Na} values. On the other hand it may be also speculated that in female cortex with worse binding properties for substrate and cofactor sodium (as indicated by higher K_m and K_{Na} values) the Na,K-ATPase functioning may be compensated by enhanced protein expression. However, from the point of energy utilization by hydrolysis of ATP the significant difference observed throughout the whole concentration range but especially in lower concentrations of ATP seems to be important when comparing cortex with cerebellum. The lower range of ATP concentrations corresponds to physiological intracellular presence of energy substrate.

The observed discrepancy between the increased expression of Na,K-ATPase catalytic subunits and lowered enzyme activity in cerebellum as compared to cortex in females might be ascribed to influence of estradiol. It is known that in cerebellum local synthesis of estradiol occurs (44, 48). So, the lower enzyme activity may me be explained based on the fact that estradiol inhibits the activity of Na,K-ATPase in the brain (36, 51-53). This observation may be of physiological relevance because there is evidence that female sex hormones like as estrogen and progesterone have ouabain-like inhibitory actions on the Na,K-ATPase (53, 54). This hypothesis is supported by various studies documenting that ovariectomy is followed by increase of Na,K-ATPase activity in the brain (55-57). Administration of estrogen to ovariectomized rats caused additional decrease of the Na,K-ATPase activity in certain parts of the brain (53).

In males the number of active Na,K-ATPase molecules was similar in both investigated regions as documented by similarity of V_{max} values. Also the binding properties for ATP and sodium were independent on the localization as indicated by similar K_m and K_{Na} values in cerebellum and in cortex. The observed localization dependent differences in expression and activities of Na,K-ATPase in cortex and cerebellum of rats point to species specific effect as in mouse brain different pattern of the enzyme expression was documented. Comparing to our data showing higher expression of α3 isoform in cerebellum, in mouse this isoform showed higher expression in cortex (58).

Point of view: sex

Concerning the sex dependent profile of Na,K-ATPase expression in cerebellum of rats, our study provided novel data showing higher presence of α1 and α3 isoforms in cerebellum of male rats, while the presence of α2 was independent on the sex. The generally higher synthesis of catalytic α subunits in cerebellum of males was reflected also in higher activities as a consequence of increased number of active enzyme molecules as indicated by upper V_{max} values.

In cortex of rats only the expression α3 isoform was higher in male group with no differences in the levels of α1 and α2 isoforms. This observation seems to be very interesting due to the fact that the α3 isoform is mostly synthesized in neurons in the central nervous system (5, 58, 59). However in the total activity of Na,K-ATPase in cortex the contribution of α1 and α2 isoforms seems to be most important as indicated by similar activities of the enzyme throughout the investigated concentration ranges for ATP or sodium in both groups of rats. In the present study the whole tissue of selected brain regions was studied. Thus, the obtained results beside the neuronal Na,K-ATPase involve also the contribution of the enzyme localized in microvascular endothelial cells. Capillary endothelial cells forming the BBB play an important role in fluid and ion homeostasis in the brain (60). It was documented that, in brain capillaries, the Na,K-ATPase is localized selectively in the antiluminal membrane part of endothelial cells (61-63). So, the data obtained in our study may indicate localization and sex dependent variations in functioning of cerebral Na,K-ATPase in neurons, as well as in microvascular endothelial cells.

To summarize, the whole tissue of selected brain regions was studied in our present study. Thus, the obtained results beside the neuronal Na,K-ATPase involve also the contribution of the enzyme localized in microvascular endothelial cells. So, the data obtained in our study may indicate localization and sex dependent variations in functioning and expression of cerebral Na,K-ATPase in neurons, as well as in microvascular endothelial cells.

Author contributions: B.K. and N.V. designed the experiment; carried out the experiment and provided input in data interpretation equally.

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Conflict of interests: None declared.

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