Effect of handling on ATP utilization of cerebral Na,K-ATPase in rats with trimethyltin-induced neurodegeneration

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Abstract
Previously it was shown that for reduction of anxiety and stress of experimental animals, preventive handling seems to be one of the most effective methods. The present study was oriented on Na,K-ATPase, a key enzyme for maintaining proper concentrations of intracellular sodium and potassium ions. Malfunction of this enzyme has an essential role in the development of neurodegenerative diseases. It is known that this enzyme requires approximately 50% of the energy available to the brain. Therefore in the present study utilization of the energy source ATP by Na,K-ATPase in the frontal cerebral cortex, using the method of enzyme kinetics was investigated. As a model of neurodegeneration treatment with trimethyltin (TMT) was applied. Daily handling (10 min/day) of healthy rats and rats suffering neurodegeneration induced by administration of TMT in a dose of (7.5 mg/kg), at postnatal days 60–102 altered the expression of catalytic subunits of Na,K-ATPase as well as kinetic properties of this enzyme in the frontal cerebral cortex of adult male Wistar rats. In addition to the previously published beneficial effect on spatial memory, daily treatment of rats was accompanied by improved maintenance of sodium homeostasis in the frontal cortex. The key system responsible for this process, Na,K-ATPase, was able to utilize better the energy substrate ATP. In rats, manipulation of TMT-induced neurodegeneration promoted the expression of the α2 isoform of the enzyme, which is typical for glial cells. In healthy rats, manipulation was followed by increased expression of the α3 subunit, which is typical of neurons.

Keywords Sodium pump · Brain · Neurodegeneration · Trimethyltin · Handling

Introduction
In the brain, the intracellular homeostasis of sodium and potassium ions is essential for regulating neuronal excitability and for multiple cellular functions. In the maintenance of proper gradient of sodium ions across the cell surface membrane, the pivotal role is ascribed to Na,K-ATPase, called also sodium pump. This enzyme located in the plasma membrane exchanges 3 intracellular ions of sodium for 2 extracellular potassium ions consuming the energy of one molecule of ATP [1, 2]. The enzyme consists of the catalytic α subunit ensuring the ATP hydrolysis with transmembrane transport of ions and the β subunit which is responsible for the correct embedding of the sodium pump into the surface membrane. Three isoforms of catalytic α subunit (α1–α3) are generally recognized in the brain [3]. Malfunction of Na,K-ATPase in the brain is probably involved in the development of several neurological disorders. Postmortem studies of the Na,K-ATPase showed alterations in the brains of patients suffering from various encephalopathies, like Alzheimer’s disease [4, 5], epilepsy [6], or bipolar disease [7]. For getting deeper insight into the molecular basis of neurodegeneration various experimental animal models were introduced. Mutation of the α3-subunit contributed to the development of rapid-onset dystonia–parkinsonism symptoms in mice [8]. In the experimental model of aluminum chloride-induced Alzheimer’s disease abnormal increase of Na,K-ATPase activity in the hippocampus of rats was observed [9]. On the other side, decreased activity of the enzyme was connected
to experimentally induced epilepsy in rodents [10, 11]. In
another model of neurodegeneration induced by administra-
tion of trimethyltin (TMT) the decrease of Na,K-ATPase
activity may be implicated as documented by inhibition of
the enzyme in synaptosomes from the forebrain of mice
when measuring the direct effect of TMT in vitro [12].
When studying the influence of various interventions on
experimental animals for the reduction of anxiety and stress,
preventive handling seems to be one of the most effective
methods [13–17]. Positive effects of handling on the behav-
ior of experimental animals were accompanied with large
variability in the response of cerebral Na,K-ATPase, from
decrease to increase of the enzyme activity [18–20]. The
functionality of the Na,K-ATPase is very important also for
maintaining energy in the brain. It is known that this enzyme
requires approximately 50% of the energy available to the
brain [21]. Therefore the present study was oriented to test
the hypothesis if the handling of experimental animals may
influence the functionality and energy consumption of the
Na,K-ATPase in the brain in control as well as in pathophysi-
ological conditions. The hypothesis was verified using the
method of enzyme kinetics as an experimental tool by esti-
mation of ATP utilization by Na,K-ATPase in the brain of
rats exposed to TMT to induce neurodegeneration focusing
on the impact of handling in adulthood.

Materials and methods

Animals

Experimental animals (male Wistar rats) at the age of
7 weeks were obtained from the breeding station Dobrá
Voda (Slovak Republic, reg. No. SK CH 24016) and main-
tained in our animal care facility (12-h/12-h light/dark
cycle with free access to food and water, relative humidity
of 55 ± 10%, and temperature of 22 ± 2 °C). After 10 days
lasting quarantine, rats were randomly divided into four
experimental groups and kept (by fours) in cages with wood
shavings and in environment enriched with paper rolls. All
procedures with animals were performed in compliance with
principles of laboratory animal care issued by EU Directive
2010/63/EU for animal experiments, proved and controlled
by the State Veterinary and Food Administration of Slovak
Republic (No. 4030/10-221), and with the agreement of the
Ethical Committee of the Center of Experimental Medicine,
Slovak Academy of Sciences.

Experimental groups

The animals (n = 32) were randomly divided into four
experimental groups: CN group: non-handled control rats
(n = 8); CH group: handled control rats (n = 8); TN group:
non-handled with TMT (n = 8); TH group: handled rats with
TMT (n = 8).

TMT administration

Rats exposed to TMT show behavioral, biochemical, and
histological deficits [22, 23]. This potent neurotoxin affects
various regions of the central nervous system, including
the neocortex, the cerebellum, and the hippocampus. At
12 weeks of age, TMT rats received a single i.p. dose of
TMT chloride (Sigma-Aldrich; 7.5 mg/kg dissolved in 0.1%
DMSO in the volume of 0.2 mL/100 g of rat body weight).
Control rats received a single i.p. dose of saline (with 0.1%
DMSO). Adult rats weighed 285 ± 5 g at the time of TMT/
saline administration.

Handling process

Six weeks lasting handling (10 min/daily) was performed
during postpartum days 60–102, as it was described previ-
ously [17]. First four weeks the handling was applied prior
to any treatment of rats and was prolonged for other 2 weeks
after TMT or vehicle administration. The same person
manipulated the rats in the van, took them to the hands, laid
them on chest, or let them climb out of the van allowing rats
to explore the surroundings of the table. Non-handled rats
were placed in separate room and without the possibility of
getting out of the cage. After six weeks of handling proce-
dure, the animals were left for additional two weeks with-
out any experimental manipulation. Experimental design
is shown in Fig. 1. Terminating the experiment, animals
were anesthetized by ether and were sacrificed in uncon-
scious state by decapitation. Frontal cortexes of the brains
were promptly removed, rapidly rinsed with ice-cold physi-
ological saline, immediately frozen in liquid nitrogen, and
stored at − 60 °C until used.

Preparation of tissue fraction for electrophoresis and
immunochemical Western blot analysis

Brain frontal cortex samples were re-suspended in homoge-
nizing buffer (50 mmol l⁻¹ Tris–HCl, 250 mmol l⁻¹ sucrose,
1 mmol l⁻¹ dithiothreitol, 1 mmol l⁻¹ phenylmethylsulfo-
nyl fluoride; pH 7.4) and homogenized with a piston-hand
homogenizer in ice, followed by ultrasonic homogenization
(UP100H, Hielscher Ultrasoundics, Germany) in repetitions
3 × 5 s in ice. The homogenates were centrifuged (Eppen-
dorf centrifuge 5804 R, Eppendorf, Germany) at 800 × g
for 5 min at 4 °C. After that, pellets were discarded and the
supernatants were centrifuged again (16,100 × g, 30 min.,
4 °C). Following this second centrifugation, the super-
натants were discarded and the pellets were re-suspended in
homogenizing buffer complemented with 0.2% Triton X-100
and centrifuged again (16,100×g, 5 min., 4 °C). The Triton X-100 soluble supernatants represented the membrane fraction. The protein concentrations were estimated by the method of Bradford [24].

Prepared samples of membrane fractions were plated and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in the equivalent volume corresponding to 30 μg protein per lane. Separated proteins were transferred from gel to a nitrocellulose membrane overnight at 4 °C (Biorad Mini-PROTEAN, USA). The quality of the transfer was controlled by Ponceau S staining of nitrocellulose membranes after the transfer. Specific primary antibodies against α1 (mouse monoclonal antibody, Sigma, A-277, in dilution 1:250, RRID:AB_258030), α2 (polyclonal antibody, Millipore, # 07-674, in dilution 1:1000, RRID:AB_390164), and α3 (rabbit polyclonal antibody, Millipore, # 06-172, in dilution 1:1000, RRID:AB_11213338) subunits of Na,K-ATPase were used for the primary immunodetection. The secondary antibodies used were peroxidase-labeled anti-rabbit (Cell Signaling, #7074S, in dilution 1:1000, RRID:AB_2099233) and anti-mouse (Cell Signaling, #7076S, in dilution 1:1000, RRID:AB_330924) immunoglobulins. Bound antibodies were detected by the enhanced chemiluminescence detection method (Amersham Imager 600, UK). Densitometric quantification of protein levels was performed by comparison to loading control beta-actin (mouse monoclonal antibody, Abcam, ab6276, in dilution 1:1000, RRID:AB_2223210) using a ImageJ software (RRID:SCR_003070).

Estimation of ATP utilization by Na,K-ATPase using the method of enzyme kinetics

For Na,K-ATPase activity assay, the tissue from frontal cortices was re-suspended and homogenized in ice-cold 50 mM Tris (pH 7.4) for 3×5 s (Polytron PT-20, speed 3) in an ice bath. Subsequently, a glass teflon homogenizer was used for finer homogenization. The amount of proteins was determined by the procedure of Lowry [25] using bovine serum albumin as a standard. All assays of the Na,K-ATPase activity were performed at 37 ºC using 20 µg  ml⁻¹ of protein from homogenate in an assay buffer containing (in mmol  l⁻¹) 4 MgCl₂, 100 NaCl, 10 KCl, and 50 TRIS (pH 7.4). The samples were pre-incubated for 20 min in a 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 trichloroacetic acid. The inorganic phosphorus generated from ATP hydrolysis was estimated according to Taussky and Shorr [26]. To establish the
enzyme activity, the specific inhibitor of the Na,K-ATPase ouabain in concentration of 3 mmol l$^{-1}$ was applied. Kinetic parameters $V_{\text{max}}$ and $K_m$ were evaluated according to the Michaelis–Menten equation from the data by direct nonlinear regression and Lineweaver–Burk method. The parameter $V_{\text{max}}$ represents the maximal velocity of enzyme reaction, and $K_m$ value refers to the concentration of energy substrate ATP necessary for half-maximal activation of the enzyme.

**Statistical analysis**

All investigated parameters are expressed as means ± standard errors of the mean (SEM). Two-way ANOVA and Holm–Sidak tests were used for statistical analysis in Sigma plot software (RRID:SCR_003210). The differences were considered to be significant when the $p$-value was less than 0.05.

**Results**

Studying the influence of TMT administration in vivo on the Na,K-ATPase in brain homogenate by activation of the enzyme with increasing concentrations of ATP we observed higher activities in the whole concentration range in non-handled TMT rats (TN) when comparing to non-handled controls (CN) (Fig. 2A). The difference decreased stepwise with increasing concentrations of substrate from 28% observed in the presence of 0.16 mmol l$^{-1}$ of ATP to 8% in the presence of 8 mmol l$^{-1}$. Analysis of the data according to the Michaelis–Menten equation resulted in significantly lowered $K_m$ value by 22% in the TN group without significant changes in the $V_{\text{max}}$ value as presented in Figs. 2B and 3.

Studying the influence of handling on the Na,K-ATPase in control rats without TMT-treatment we observed higher activities especially in the lower concentration range of ATP in handled control rats (CH) when comparing to non-handled controls (CN) (Fig. 2A). The highest increase in activity amounting 48% was observed in the presence of 0.16 mmol l$^{-1}$ of ATP and it decreased stepwise with increasing concentrations of substrate. In the presence of ATP above 2.4 mmol l$^{-1}$ of ATP the enzyme activity was

![Fig. 2](image_url)

**Fig. 2** A: Activation of the Na,K-ATPase by low concentrations of substrate ATP in control non-handled rats (CN), in control handled rats (CH), in non-handled rats with TMT administration (TN), and in handled rats with TMT administration (TH). Inset: activation of the enzyme in the whole investigated concentration range of ATP. Data represent mean ± SEM, $n=8$ in each group. B: Transformation of the data from kinetic measurements to Lineweaver–Burk plot.

![Fig. 3](image_url)

**Fig. 3** Kinetic parameters of the Na,K-ATPase during activation with substrate ATP in control non-handled rats (CN), in control handled rats (CH), in non-handled rats with TMT administration (TN), and in handled rats with TMT administration (TH). The parameter $V_{\text{max}}$ 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, $n=8$ in each group. Significance a: $p<0.001$ vs. CN, b: $p<0.05$ vs. CN, c: $p<0.001$ vs. TN.
similar in both control groups without TMT. Analysis of the data resulted in significantly lower \( K_m \) value by 47% in the CH, as compared to the CN group without significant changes in the \( V_{\text{max}} \) value (Figs. 2B and 3).

In animals treated with TMT the handling induced also increase of Na,K-ATPase activity in the lower concentration range of ATP when comparing to non-handled TMT-treated rats (TN) (Fig. 2A). The highest increase amounted to 25% in the presence of 0.16 mmol l\(^{-1}\) of ATP and it decreased stepwise with increasing concentrations of substrate. In the presence of ATP above 0.8 mmol l\(^{-1}\) of ATP the enzyme activity was similar as compared to the non-handled group with TMT. Analysis of the data resulted in significantly lower \( K_m \) value by 37% in the TH, as compared to the TN group without significant changes in the \( V_{\text{max}} \) value (Figs. 2B and 3). Statistical analysis by two-way ANOVA indicates no significant effect of handling concerning the \( V_{\text{max}} \) value in all four experimental groups. The \( K_m \) values were significantly lowered in both handled groups of control animals as well as in TMT-treated animals.

Investigation of the protein expression of catalytic \( \alpha \) subunits of Na,K-ATPase by Western blot showed that \( \alpha_1, \alpha_2, \) and \( \alpha_3 \) subunits were affected variously in the frontal cortex of handled or non-handled, and TMT or saline-affected rats (Table 1). While the expression of \( \alpha_1 \) subunit of Na,K-ATPase remained unaffected after TMT treatment as well as after handling, the expression of \( \alpha_2 \) subunit was higher after regular daily handling of experimental animals in rats subjected to TMT as compared to non-handled rats with TMT. The TMT did not significantly affect the expression of \( \alpha_2 \) subunit in non-handled control animals. Concerning the expression of \( \alpha_3 \) subunit, significant influence of handling was observed resulting in higher protein expression in the CH control group when compared to the CN group (Table 1).

### Discussion

Studies devoted to various neurodegenerative disorders pointed out to malfunction of certain isoforms of the Na,K-ATPase in the brain. Due to the presence of 3 various isoforms of the catalytic \( \alpha \) subunit in the brain tissue the data obtained by our investigation of enzyme kinetics represent the cumulative activity of all 3 isoforms of the enzyme. Previously it was documented that administration of TMT to rats induced neurodegeneration [27]. By in vitro studies, it was shown that TMT was able to inhibit the Na,K-ATPase in synaptosomes from the forebrain of mice [12] indicating that in the neurotoxic action of trimethyltin (TMT) this enzyme might be involved. Moreover, recently it was documented that TMT in dose-dependent mode decreased the activity of Na,K-ATPase and its expression in the hippocampus and medulla oblongata of mice [28]. In the present study we tried to broaden the information concerning the molecular principles of expected alterations of Na,K-ATPase in vivo in rats, four weeks after administration of TMT. Our investigations using measurements of enzyme kinetics showing higher Na,K-ATPase activities in the lower concentration range of ATP indicate better utilization of the energy substrate confirmed also by the lowered value of \( K_m \) in TMT-treated non-handled animals when compared to non-handled healthy controls. The number of active Na,K-ATPase molecules remained unchanged as indicated by similar values of \( V_{\text{max}} \) in TMT and the control group, both without handling. So the effect of TMT on the Na,K-ATPase from the frontal cortex seems to be different when measured in vitro and in vivo. Our measurement four weeks after in vivo administration of TMT showed higher effect in the presence of physiologically relevant concentrations of ATP (0.16–0.80 mmol l\(^{-1}\)), while the in vitro measurements of Costa [12] were done at higher concentration (2 mmol l\(^{-1}\)). Another important fact probably contributing to the difference between in vivo and in vitro experiments is the probably reduced presence of TMT due to urinary excretion of TMT during the in vivo experiment. Previously, it was documented that after 8 days the TMT presence decreased almost by 50% in mice and rats [29].

### Table 1

|          | CN Control non-handled | CH Control handled | TN TMT non-handled | TH TMT handled |
|----------|------------------------|--------------------|--------------------|---------------|
| \( \alpha_1 \) | 100±11.3               | 109.1±10.7         | 90.2±5.5           | 116.5±13.6    |
| \( \alpha_2 \) | 100±5.8                | 114.5±11.2         | 98.2±5.3           | 182.3±18.7\(^{a} \) |
| \( \alpha_3 \) | 100±3.5                | 135.4±13.2\(^{b} \) | 107.9±11.8         | 124.5±8.1     |

Data represent mean±SEM, \( n = 5 \) in each group

Significance: \( a: p < 0.05 \) vs. CH and TN, \( b: p < 0.05 \) vs. CN
Previous study of TMT-induced neurodegeneration showed that handling of young adult male rats improved the spatial memory in healthy and also in TMT-treated rats and prevented the memory impairment induced by trimethyltin [17]. Therefore the second aim of the present study was oriented to the effect of handling of rats on the Na,K-ATPase properties in healthy rats and also in rats with TMT-induced neurodegeneration.

Our present results of enzyme kinetic investigations indicate that the Na,K-ATPase might be involved in positive effects of handling in healthy rats as well as in rats with TMT-induced neurodegeneration. In both groups of handled rats the number of active enzyme molecules remained unchanged, as indicated by unaltered $V_{\text{max}}$ value when comparing to respective control. On the other side, the ability of the enzyme to bind and utilize the energy substrate ATP was markedly improved as indicated by lowered $K_m$ values. So, the handling of rats may affect similarly the energy utilization and extrusion of superfluous sodium from cells in the frontal cortex in healthy rats and in rats with TMT-induced neurodegeneration, securing thus better comfort of experimental subjects. It may be hypothesized that attaining better comfort may contribute to improved behavior of studied subjects independently on their pathophysiological status as it was shown previously [17, 30, 31].

The positive impact of enhanced Na,K-ATPase functionality in the brain tissue probably plays an important role in the improvement of spatial memory in healthy non-treated rats and rats with TMT-induced neurodegeneration when handled for 6 weeks. The importance of Na,K-ATPase in the prevention of neurodegeneration was supported also in the experimental model of epilepsy where stimulation of Na,K-ATPase with specific antibody (DRRSAb) restored the crossing activity of pilocarpine-treated mice in the open field test [32].

This study provides also new information concerning the expression of individual $\alpha$ subunits of Na,K-ATPase ($\alpha1$, $\alpha2$, $\alpha3$) in the frontal cerebral cortex in consequence of handling of healthy rats or rats with TMT-induced neurodegeneration. The global maintenance of intracellular sodium homeostasis was probably not affected by TMT treatment of adult rats in handled as well as in non-handled rats as indicated by unaltered expression of $\alpha1$ subunit. This ubiquitously expressed isofrom is responsible for the global maintenance of intracellular sodium homeostasis [33, 34].

The increased expression of glial isofrom $\alpha2$ in handled rats with TMT-induced neurodegeneration seems to be interesting in view of pathological alterations in consequence of poisoning by TMT. It was previously documented that malfunction of this isofrom of Na,K-ATPase is probably involved in the development of familial hemiplegic migraine type 2 [35]. However, the increased expression of glial isofrom $\alpha2$ in TMT affected and handled rats may represent an interesting effect requiring further investigations for explaining the physiological relevance of this fact.

Based on our analysis of the protein expression of Na,K-ATPase $\alpha3$ subunit it may be hypothesized that increased expression of this subunit could play a role in the neuroprotective mechanism of handling in healthy rats that were not poisoned by TMT. Previously it was documented that $\alpha3$ subunit is exclusively specific for fully differentiated neurons [36], thus we can suppose that handling of healthy animals might act at the neuronal level. Na,K-ATPase stimulates the growth of dendrites during the development of the brain where signal growth is triggered by signal transduction and probably plays a role in neurogenesis [37]. Malfunction of $\alpha3$ is probably involved in the development of several neurological disorders, as it was suggested also for rapid-onset dystonia–parkinsonism symptoms in mice [8]. So, it may be hypothesized that increased expression of neuronal $\alpha3$ probably is involved in the mechanism of previously reported improvement in memory of handled control animals [17]. This hypothesis seems to be in agreement with previous observation of altered spatial learning, motor activity, and anxiety in $\alpha3$-deficient mice [38]. In addition, the animals with the haploinsufficiency gene for this subunit of Na,K-ATPase were accompanied by cognitive deficits [39].

**Conclusion**

Based on the present results it can be concluded that everyday handling of rats, besides the previously published beneficial effect on spatial memory, and improved behavioral properties of experimental animals may be also a consequence of improved maintenance of sodium homeostasis in the frontal cortex of brains. The key system responsible for this process, the Na,K-ATPase, was able to utilize better the energy substrate ATP. In rats with TMT-induced neurodegeneration handling promoted the expression of $\alpha2$ isofrom of the enzyme which is typical for glial cells. In healthy rats, the handling was followed by increased expression of $\alpha3$ subunit which is typical for neurons.

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**Author contributions** ZG designed the study; VNS, DM, and EU performed the biological model; BK, NV, DS, and JV performed the analysis and evaluation of Na,K-ATPase expression and estimation of ATP utilization by Na,K-ATPase using the method of enzyme kinetics; ZG, BK, DS, and NV prepared the manuscript.

**Data availability** The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.
Declarations

Conflict of interest No conflicts of interest, financial, or otherwise are declared by the authors.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures with animals were performed in compliance with principles of laboratory animal care issued by EU Directive 2010/63/EU for animal experiments, proved and controlled by the State Veterinary and Food Administration of Slovak Republic (No. 4030/10-221), and with the agreement of the Ethical Committee of the Center of Experimental Medicine, Slovak Academy of Sciences. This article does not contain any studies involving human participants performed by any of the authors.

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