

Functional Interaction Between Na/K-ATPase and NMDA Receptor in Cerebellar Neurons

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Received: 26 July 2014 / Accepted: 29 September 2014 / Published online: 8 November 2014
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Abstract NMDA receptors play a crucial role in regulating synaptic plasticity and memory. Activation of NMDA receptors changes intracellular concentrations of Na^+ and K^+ , which are subsequently restored by Na/K-ATPase. We used immunohistochemical and biochemical methods to elucidate the potential mechanisms of interaction between these two proteins. We observed that NMDA receptor and Na/K-ATPase interact with each other and this interaction was shown for both isoforms of α subunit ($\alpha 1$ and $\alpha 3$) of Na/K-ATPase expressed in neurons.

Using Western blotting, we showed that long-term exposure of the primary culture of cerebellar neurons to nanomolar concentrations of ouabain (a cardiotonic steroid, a specific ligand of Na/K-ATPase) leads to a decrease in the levels of NMDA receptors which is likely mediated by the $\alpha 3$ subunit of Na/K-ATPase. We also observed a decrease in enzymatic activity of the $\alpha 1$ subunit of Na/K-ATPase caused by NMDA receptor activation. This effect is mediated by an increase in intracellular Ca^{2+} . Thus, Na/K-ATPase and NMDA receptor can interact functionally by forming a macromolecular complex which can be important for restoring ionic balance after neuronal excitation. Furthermore, this interaction suggests that NMDA receptor function can be regulated by endogenous cardiotonic steroids which recently have been found in cerebrospinal fluid or by pharmacological drugs affecting Na/K-ATPase function.

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Keywords Na/K-ATPase · NMDA receptors · Calcium ·
Ouabain · Neurons · Primary culture of cerebellar neurons

Introduction

Na/K-ATPase (the sodium pump) is an integral membrane protein expressed in all mammalian cells. It maintains the asymmetric distribution of Na^+ and K^+ ions across the cell membrane by actively exporting three Na^+ and importing two K^+ while hydrolyzing one ATP molecule [1]. Na/K-ATPase is responsible for approximately 50 % of total brain energy consumption [2]. In neurons, the catalytic α subunit of Na/K-ATPase occurs in two isoforms, the ubiquitous $\alpha 1$ subunit and the neuron-specific $\alpha 3$ subunit [3, 4]. Mutations in the gene ATP1A3 encoding the $\alpha 3$ subunit are associated with two syndromes: rapid-onset dystonia-parkinsonism [5] and alternating hemiplegia of childhood [6].

Na/K-ATPase is a specific receptor for cardiotoxic steroids (CTS), including ouabain [7]. Ouabain and at least two other CTS, digoxin and marinobufagenin, have been found in blood, adrenals, and hypothalamus. Circulating ouabain is bound by a specific binding globulin [8]. The concentration of CTS can be physiologically modulated through physical exercise and hypoxia [9]. In rodents, $\alpha 3$ is 10^3 times more sensitive to ouabain than $\alpha 1$.

Na/K-ATPase has been shown to interact with several proteins, and there is evidence that binding of CTS to Na/K-ATPase can modulate functions of its partners in protein-protein interaction [10–13]. Previous studies have suggested that there may be functional interactions between Na/K-ATPase and NMDA receptor [14]; however, the mechanism of this interaction remains unclear. NMDA receptor is an ionotropic glutamate receptor. Its distinctive feature in comparison to other ionotropic glutamate receptors is permeability to calcium (in addition to Na^+ and K^+), which is an important secondary messenger capable of modulating cellular response according to the external signal [15]. It forms a heterotetramer between two obligatory NR1 subunits and two NR2 subunits which occur in four isoforms (A–D) [16]. Recent studies have suggested that CTS can affect NMDA receptors [17–19], which participate in synaptic plasticity and molecular memory formation [15, 16]. It has been reported that administration of the CTS endobain E for 2 days leads to an increase in NMDA receptor expression in the cortex and hippocampus [20]. At the same time, mice heterozygous for the $\alpha 3$ subunit displayed a 40 % reduction in hippocampal NMDA receptor expression [21]. No proteomic studies have demonstrated direct interaction between Na/K-ATPase and NMDA receptor, but NMDA receptors have been shown to interact with PSD-95, PLC γ , PI3K, and tubulin [22], which are also known to interact with the α subunit of Na/K-ATPase [23, 12, 24–26]. Taken together, these observations prompted us to explore the mechanism of interaction between Na/K-ATPase and NMDA receptor and to study whether and to what extent such an interaction may be modulated by ouabain and/or by NMDA receptor ligands.

This study has been performed on rat primary cerebellar neurons. Protein-protein interaction was assessed with co-immunoprecipitation. Functional interaction was studied by immunochemical and biochemical methods in the presence and absence of NMDA or ouabain in concentrations that would discriminate between the effects on $\alpha 3$ and $\alpha 1$ isoforms.

Materials and Methods

Materials

Chemicals (≥ 95 % purity) were purchased from Sigma (St Louis, MO). Regents for cell culture, and culture media were

purchased from Invitrogen (Carlsbad, CA). Antibodies against NR1 (sc-9058), NR2B (sc-9057) subunits of NMDA receptor, goat anti-mouse IgG-horseradish peroxidase, and goat anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against tubulin (32–2500) were purchased from Invitrogen (Camarillo, CA). Antibodies against $\alpha 1$ subunit of Na/K-ATPase ($\alpha 6F$) were purchased from Developmental Studies Hybridoma Bank (Iowa City, IA). Antibodies against $\alpha 3$ subunit of Na/K-ATPase (MA3-915) were purchased from Pierce Biotechnology (Rockford, IL).

All research on rats were done in USA according to procedures and guidelines of the National Institutes of Health, and the protocols were approved by the Institutional Animal Care and Use Committee of the University of Toledo, College of Medicine and Life Sciences. Animal experiments in Russia were approved by the Institutional Committee for Ethics in Animal Experimentation of Biological Faculty, Moscow State University, Moscow, Russia.

Primary Culture of Cerebellar Neurons

Primary cultures of cerebellar neurons were prepared as follows [27]. Cerebella from 3–5-day-old Wistar rats of both sexes were excised, washed in cold HBSS (Invitrogen), and cut into small pieces with a scalpel. Tissue was suspended in 0.05 % Trypsin-EDTA and incubated at 37 °C for 20 min. This reaction was stopped by fresh MEM with 10 % FBS. Next, tissue was washed two times by warm HBSS and then transferred to 5 ml of Neurobasal-A Medium and triturated with a series of flame-polished glass Pasteur pipettes of decreasing tip diameter. The cell suspension was centrifuged at 300 g for 2 min, and the pellet was resuspended in culture medium consisting of Neurobasal-A Medium supplemented with 2 % B-27, 0.5 mM GlutaMax, 20 mM KCl, and 100 U/ml penicillin/streptomycin. Neurons were plated at a density of $5 \cdot 10^5$ cells/cm² onto 6-well or 12-well plates pretreated with poly-D-lysine. Cultures were incubated in culture medium and maintained in a humidified atmosphere at 37 °C in 5 % CO₂. Experiments were performed after 11 days.

Immunoprecipitation

For immunoprecipitation experiments, neurons were treated with ice-cold lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 % Triton X-100, 60 mM octylglucoside, 1 mM PMSF, 1 mM Na₃VO₃, 1 mM NaF, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. After 30 min at 4 °C, the lysate was clarified by centrifugation at 24000g for 15 min. The supernatant (0.25–1 mg protein) was precleared and incubated with the appropriate antibody and then with protein A plus

agarose beads. The proteins bound to the collected beads were subjected to SDS-PAGE and probed with appropriate antibodies as indicated below.

Western Blotting Analysis

Samples were subjected to 8 or 10 % SDS-PAGE, transferred to PVDF membrane, and probed with appropriate antibodies by standard procedures. The immunoreactive bands were developed and detected using enhanced chemiluminescence. For quantitative comparisons, images were scanned with a densitometer. Different dilutions of samples were subjected to SDS-PAGE, and multiple exposures of the films were used to ensure that quantifications were made within the linear range of the assays.

Na/K-ATPase Activity

Na/K-ATPase activity was measured as described previously [28]. Liberated inorganic phosphate was measured after the enzymatic reaction. This assay was performed in the presence and absence of 1 mM ouabain, and the ouabain-sensitive component was considered Na/K-ATPase activity. After treatment, cells were permeabilized by five freeze-thaw cycles, the enzymatic reaction was conducted, and the Rathbun and Betlach method was used for measuring inorganic phosphate. The Lowry protein assay was used to normalize the samples.

Fluorescence Microscopy

Treated cells were fixed in 3.7 % paraformaldehyde for 10 min at RT or in 10 % TCA for 5 min at 4 °C. Cells were then washed three times with PBS and permeabilized with 0.05 % Triton X-100 for 5 min at RT. Following three more washes with PBS, cells were blocked with 10 % BSA and incubated with primary antibodies overnight at 4 °C. After incubation, cells were washed three times with PBS and incubated with secondary antibodies for 1 h in the dark at RT. Coverslips were mounted with ProLong Gold antifade reagent (Molecular Probes). Images were obtained using a Leica TCS SP5 broadband confocal microscope system equipped with Argon and HeNe lasers coupled to a DMI 6000CS inverted microscope with a $\times 63$ oil immersion objective. Alexa Fluor 488 was excited using 488 spectral laser lines. The Leica Confocal Microscope System software was used for visualization and analysis.

MTT Test

Neuronal viability was assessed using MTT test. The method is based on reduction in the living cells of yellow

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to blue formazan. Cultures used in experiments were planted into 96-well plates with a density of 4×10^4 cells per well. Following experimental procedures, a solution of MTT in NBM was added to each well to obtain a final MTT concentration of 0.5 mg/ml. Wells without cells were used as negative control. After incubation with MTT for 2–3 h, the medium was completely removed from the wells and 100 μ l of DMSO were added to each well. Sample absorbance was measured at wavelengths 570 and 660 nm using plate reader Synergy H4 (BioTek). Absorbance values at 660 nm as well as that in the negative control were subtracted from absorbance values at 570 nm. Data were presented as a percentage of the signal in the control wells (without ouabain or NMDA).

Analysis of Data

Values are means \pm SE of the results of a minimum of three experiments. Student's *t* test was used and significance was accepted at $p < 0.05$.

Results

Analysis of Structural Interaction Between Na/K-ATPase and NMDA Receptor

First, we observed that the primary culture of rat cerebellar neurons we used contained both ouabain-sensitive $\alpha 3$ and ouabain-resistant $\alpha 1$ subunits of Na/K-ATPase by immunostaining (Fig. 1a–b), which is consistent with the previous literature [4]. Also, we showed that the primary culture of rat cerebellar neurons contained both NR1 and NR2B subunits of NMDA receptor by Western blots (Fig. 1c, d).

Then, we evaluated possible protein interactions using co-immunoprecipitation. In a series of experiments, we incubated cerebellar neurons with 1 μ M ouabain for 10 min, then lysed the cells and performed co-immunoprecipitation using antibodies against either NR1 or NR2B subunits. Na/K-ATPase and NMDA receptor molecules were solubilized under the conditions similar to the literature [23, 29]. As shown as Fig. 2, both $\alpha 1$ and $\alpha 3$ subunits co-immunoprecipitate with NMDA receptor. Thus, both subunits form a complex with NMDA receptor and potentially can participate in glutamate exchange regulation mechanisms. However, the specific ligand of Na/K-ATPase, ouabain [7, 30], did not cause significant changes in the interaction of NMDA receptor and Na/K-ATPase α subunits,

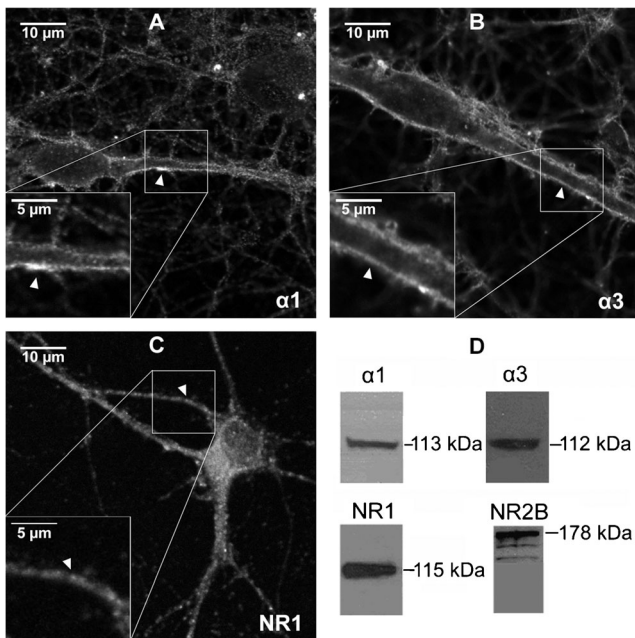


Fig. 1 Presence of $\alpha 1$ and $\alpha 3$ subunits of Na/K-ATPase and NR1 and NR2B subunits of NMDA receptor in primary culture of cerebellar neurons. Neurons were stained with antibodies against $\alpha 1$ (a) and $\alpha 3$ (b) subunits of Na/K-ATPase and NR1 subunit of NMDA receptor (c). **d** Representative Western blots showing the presence of $\alpha 1$ and $\alpha 3$ subunits of Na/K-ATPase and NR1 and NR2B subunits of NMDA receptor in cerebellar neurons

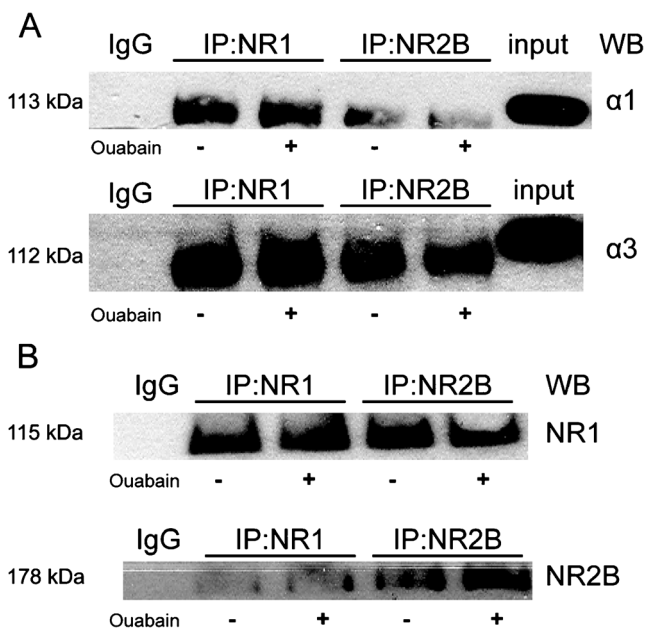


Fig. 2 Co-immunoprecipitation of NR1 and NR2B subunits of NMDA receptor with $\alpha 1$ and $\alpha 3$ subunits of Na/K-ATPase. Cell lysates of primary culture of cerebellar neurons were immunoprecipitated (IP) with monoclonal antibodies against NR1 or NR2B subunits of NMDA receptor and then probed by Western blotting with antibodies against $\alpha 1$ and $\alpha 3$ of Na/K-ATPase (a) and NR1 and NR2B subunits of NMDA receptor (b). One representative blots from three independent experiments

indicating this interaction does not affect by ouabain binding of Na/K-ATPase.

The Effects of Long-Term Exposure of Neurons to Ouabain

We tested whether exposure of cerebellar neurons to ouabain affects the amount of NMDA receptor. We incubated cells with 1 μ M ouabain for periods of time from 0.5 to 6 h (Fig. 3a), then lysed the cells and analyzed the amount of NR1 and NR2B subunits of NMDA receptor as well as $\alpha 1$ and $\alpha 3$ subunits of Na/K-ATPase.

The results showed that long-term incubation with ouabain (1 h or more) causes about 40 % decrease in the amount of NMDA receptor subunits. Furthermore, the level of NR2B subunit decreases faster than that of NR1 subunit. Thus, we can assert that ouabain causes a decrease in the number of NMDA receptors because NR1 is an obligatory subunit of NMDA receptor [16]. Notably, after 6 h incubation with 1 μ M ouabain, the amount of NMDA receptors did not change further (data not shown). We did not observe significant change in the levels of $\alpha 1$ and $\alpha 3$ subunits of Na/K-ATPase at 1 μ M ouabain (data not shown). To identify which Na/K-ATPase isoforms is involved in this process, we tested different ouabain concentration (ranging from 1 nM to 10 μ M) after 6 h incubation (Fig. 3b). We did not see changes in the amount of Na/K-ATPase α subunits by lower than 10 μ M ouabain (data not shown).

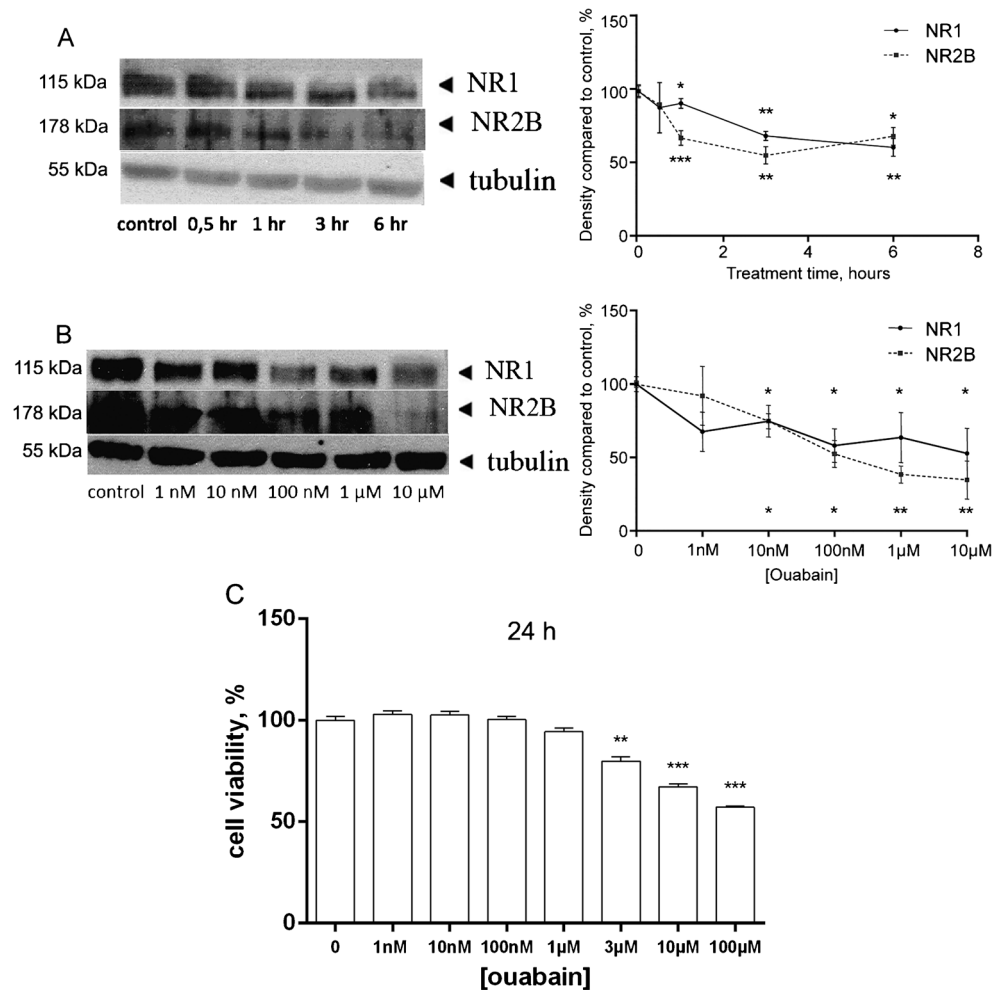
Data show the reduction of NR1 subunit decreases at only 1 nM ouabain, and further increase in ouabain concentration does not intensify this effect. The amount of NR2B subunit is dose-dependent in the range of ouabain concentrations from 1 nM to 1 μ M, reaching the maximum effect of less than 50 % of the control value at 1 μ M ouabain. Further increase in ouabain concentration does not significantly intensify the decrease in NR2B amount.

Apart from that, we tested whether ouabain in concentrations used in our experiments caused neuronal death. We analyzed the effect of ouabain on neuronal viability using MTT test after 24-h incubation with various ouabain concentrations (Fig. 3c). We found that ouabain in concentrations of 1 μ M or less did not cause cell death. Thus, the decrease in NMDA receptor levels caused by 1 nM–1 μ M ouabain cannot be explained to be induced due to neuronal death.

We conclude that ouabain binding to the ouabain-sensitive $\alpha 3$ subunit of Na/K-ATPase (ouabain concentrations ranging from 1 to 100 nM) leads to a decrease in NMDA receptor level, while ouabain binding to the ouabain-resistant $\alpha 1$ subunit (1–100 μ M ouabain) has no effect on this process because ouabain in concentrations 100 nM–100 μ M does not significantly enhance the effect.

Fig. 3 Treatment with ouabain causes a decrease in the amount of NMDA receptor. Primary cultures of cerebellar neurons were incubated with 1 μM ouabain for different periods of time (a) or with different concentrations of ouabain for 6 h (b).

Representative Western blots showing reduced levels of NR1 and NR2B in primary culture of cerebellar neurons. The optical density of the control was taken for 100 %; data were normalized to tubulin as a loading control. c Primary cultures of cerebellar neurons were incubated with ouabain for 24 h, then MTT tests were conducted as described in “Materials and Methods”. The viability in the control wells was taken for 100 %. * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control, *** $p < 0.001$ compared to control



NMDA Receptor Regulates Na/K-ATPase Enzymatic Activity

Previously, it has been shown that NMDA receptor activation can cause a decrease in Na/K-ATPase activity in cell lysates [14]. To further test this mechanism, we first studied the dose-dependent effects of NMDA ranging from 5 to 750 μM on Na/K-ATPase's enzymatic activity in primary neuronal cultures (Fig. 4a). When neurons were incubated with different concentrations of NMDA for 30 min, neurons were permeabilized and Na/K-ATPase enzymatic activity was measured. While previous literature shows that NMDA receptor activation by NMDA in concentrations above 0.1 mM leads to a dose-dependent decrease in Na/K-ATPase activity [14, 31], we observed a small increase in Na/K-ATPase activity at 10–25 μM NMDA with significant decrease at higher doses (more than 0.1 mM).

To ensure that 30-min incubation with chosen NMDA concentrations did not affect cell viability, we conducted MTT test. Primary cultures of cerebellar neurons were incubated with NMDA for 30 min, then washed and incubated until 24 h in culture medium. Cells were incubated with

NMDA for 24 h as positive control. As shown in Fig. 4b, 30-min incubation with different NMDA concentrations up to 1 mM did not affect cell viability, while 24-h incubation with NMDA caused a significant decrease in cell viability starting from 50 μM NMDA.

To test Ca^{2+} dependence of the effect of NMDA on Na/K-ATPase activity, we preincubated cells with BAPTA (Ca^{2+} chelator), then exposed the neurons to NMDA. As shown in Fig. 4c, the effect of NMDA on Na/K-ATPase activity is prevented by preincubation with BAPTA. Importantly, BAPTA itself does not affect Na/K-ATPase activity.

Finally, to identify the isoform of Na/K-ATPase that was affected by NMDA, we conducted a dose-response curve for ouabain inhibition of Na/K-ATPase enzymatic activity in cell lysates. We also examined the changes in the curve caused by preincubation of NMDA, and with NMDA+D-AP5 (a selective NMDA receptor antagonist) before the lysis (Fig. 4d). Ouabain inhibition curve showed two phases. As the same as the previous report [32], the $\alpha 3$ subunits of Na/K-ATPase in rat neurons were inhibited by ouabain in concentrations ranging from 1 nM to 1 μM , while the $\alpha 1$ subunits were inhibited by ouabain in concentrations ranging from 1 μM to 1 mM. In

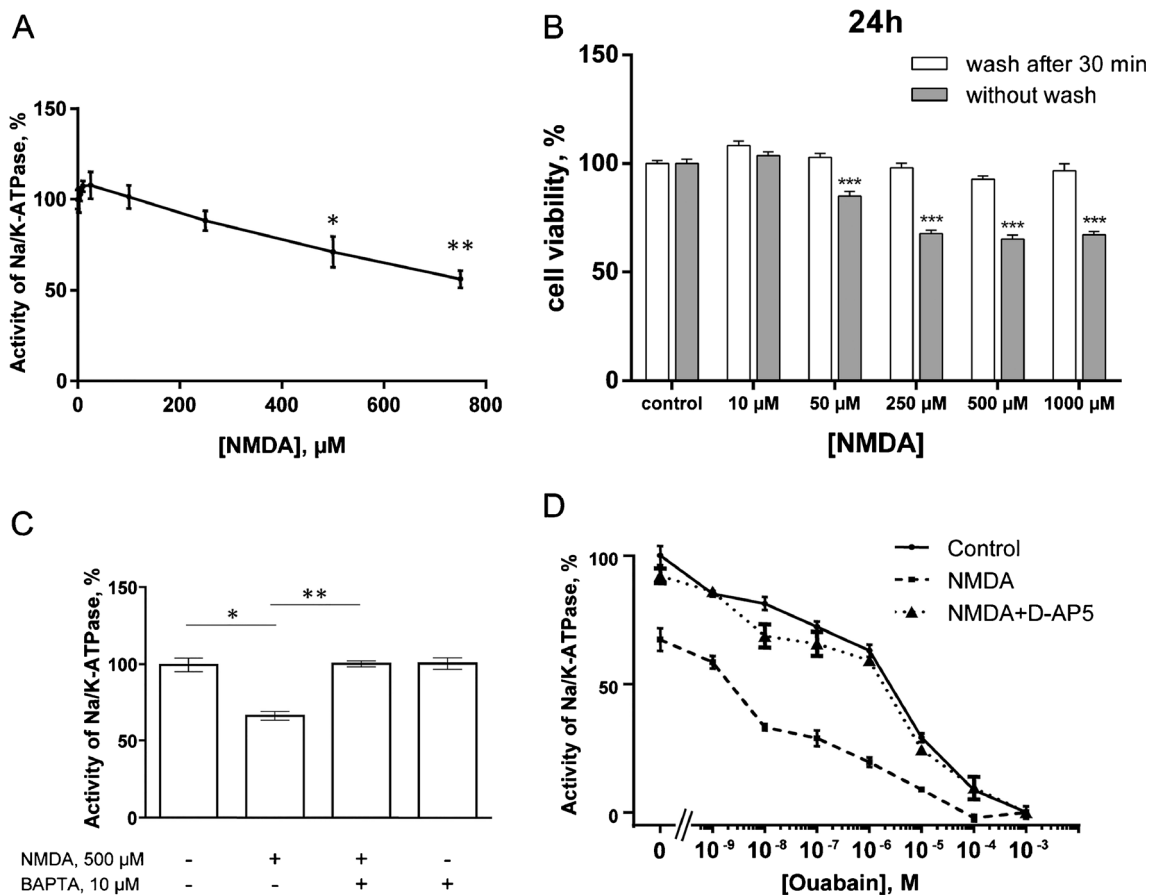


Fig. 4 Effect of NMDA treatment on Na/K-ATPase activity and cell viability in primary cultures of cerebellar neurons. **a** Effect of NMDA on Na/K-ATPase activity. Primary cultures of cerebellar neurons were incubated with different NMDA concentrations (30 min), lysed, and used to conduct the enzymatic reaction as described in “Materials and Methods”. The activity of the control was taken for 100 %; data were normalized to a measured amount of total protein. **b** Effect of NMDA on cell viability. Neurons were either incubated for 24 h with different NMDA concentrations, or incubated with NMDA for 30 min, then washed and incubated until 24 h in culture medium; then, MTT tests were conducted as described in “Materials and Methods”. The viability in the control wells was

taken for 100 %. **c** NMDA-induced decrease in Na/K-ATPase activity was prevented by BAPTA. Neurons were pre-incubated with BAPTA (10 μM, 10 min), then incubated with NMDA (500 μM, 30 min), and then lysed and used to measure Na/K-ATPase activity. **d** D-AP5 blocked NMDA inhibition on ouabain curves of Na/K-ATPase activity. Primary cultures of cerebellar neurons were pre-incubated with or without D-AP5 (10 μM, 10 min), then incubated with NMDA (500 μM, 30 min). Cells were lysed and used to conduct the enzymatic reaction. * $p < 0.05$ compared to control, ** $p < 0.01$ compared to control, *** $p < 0.001$ compared to control

our case, the activity from $\alpha 3$ subunit accounts for about 30 % of total Na/K-ATPase activity in the primary rat cerebellar neurons. As seen in Fig. 4d, the action of NMDA (either with or without D-AP5) did not cause significant changes in $\alpha 3$ subunits’ activity, because the activity still showed two phases. Ouabain-resistant $\alpha 1$ subunit activity accounts for about 70 % of total Na/K-ATPase activity. After preincubation with NMDA at 500 μM, the activity was reduced to 25 % of the total Na/K-ATPase activity compared to the control samples (without NMDA). D-AP5 prevented the effects of NMDA on $\alpha 1$ activity, so it returned to 70 % of the total Na/K-ATPase activity like it did in the control samples. Thus, we conclude that NMDA causes the inhibition of $\alpha 1$ subunit and does not affect $\alpha 3$ subunits.

Discussion

Previous studies have suggested that Na/K-ATPase and NMDA receptor can participate in functional interaction: changes in Na/K-ATPase activity can affect the properties of the NMDA receptor [18–21, 33], and NMDA receptor activation can mediate Na/K-ATPase activity [14, 34, 35]. The present study was devoted to molecular mechanisms of this interaction. First, we showed that our model system contained both the ouabain-sensitive $\alpha 3$ subunit and the ouabain-resistant $\alpha 1$ subunit of Na/K-ATPase, as well as a functional NMDA receptor. The latter was confirmed by showing the presence of both NR1 and NR2B subunits, since NR1 subunit and one of four NR2 subunits (A–D) are needed to form a functional NMDA receptor [16]. According to the literature, NR2B subunit is predominantly

expressed in the cerebellum [36]; therefore, we chose this subunit for our research.

Na/K-ATPase is involved in protein-protein interaction with various membrane and cytoplasmic proteins and is capable of affecting their properties. Such interactions could represent a general principal of regulation of various proteins by CTS binding to Na/K-ATPase [13, 37–39]. Existing data has shown that ouabain binding to Na/K-ATPase can affect the properties of proteins participating in glutamate exchange in nervous tissue, such as ionotropic glutamate AMPA receptors in neurons [40] and glutamate transporters GLT-1 and GLAST in glial cells [41]. Using co-immunoprecipitation, we demonstrated that Na/K-ATPase forms a functional complex with NMDA receptor. However, we observed that ouabain does not affect the amount of NMDA receptor molecules associated with Na/K-ATPase α subunits, indicating that the proteins interact regardless of ouabain binding to the α subunit.

This interaction was shown in both $\alpha 1$ and $\alpha 3$ subunits of Na/K-ATPase, which may indicate that a conserved domain present in both isoforms is responsible for the interaction. Thus, likely, both isoforms can functionally interact with the NMDA receptor. Previously, it has been shown that Na/K-ATPase interacts with another ionotropic glutamate receptor—AMPA receptor [40] which shows structural homology with NMDA receptor. Co-immunoprecipitation of AMPA receptor was found only with the $\alpha 1$ but not the $\alpha 3$ subunit of Na/K-ATPase. These data allow us to believe that different sites of the α subunit of Na/K-ATPase are responsible for interaction with AMPA and NMDA receptors. Taken together, these data indicate that both $\alpha 1$ and $\alpha 3$ subunits of Na/K-ATPase are co-localized with NMDA receptors and form a functional complex either by interacting directly or through some intermediate proteins.

Furthermore, we demonstrated that long-term ouabain exposure induced regulation of NMDA receptor expression. We found that exposure of the cells to low ouabain concentrations (1 nM and higher) causes a decrease in the levels of NMDA receptor subunits (NR1 and NR2B), but these changes do not lead to the cell death. According to the literature, the ouabain-sensitive $\alpha 3$ subunit of Na/K-ATPase in rat neurons is inhibited by ouabain in concentrations ranging from 1 nM to 1 μ M, while the ouabain-resistant $\alpha 1$ subunit is inhibited by ouabain in concentrations ranging from 1 μ M to 1 mM [32]. We concluded that ouabain binding to the ouabain-sensitive $\alpha 3$ subunit of Na/K-ATPase but not the $\alpha 1$ subunit leads to a decrease in the level of NMDA receptors because this effect was caused by nanomolar concentrations of ouabain. Notably, it has been reported that endobain E injection into the lateral cerebral ventricle of rats resulted to an increase in the expression of NMDA receptor subunits in the cerebral cortex and hippocampus [20]. However, in our study, we observed a decrease in NMDA receptor levels after exposure to ouabain, which is in agreement with observations reported in Na/K-

ATPase heterozygous mice [21]. It is possible that CTS may cause different effects in various regions of the brain. Another explanation could be that endobain E and ouabain simply act differently. In light of high sensitivity of NMDA receptor in response to low ouabain concentrations, we suggest that this mechanism may represent one of the ways of regulating the glutamatergic system in the brain, since recent evidence indicates that CTS could appear as endogenous steroid hormones [8, 9]. Recently, it has been shown that 2 nM ouabain is present in human cerebrospinal fluid [42] which correlates with our data on the effect of nanomolar concentrations of ouabain. Taken together, these data indicate that ouabain can play a crucial role in regulating the amount of NMDA receptor via $\alpha 3$ subunit of Na/K-ATPase. Thus, it is possible that ouabain might function as an endogenous regulator of the glutamatergic system. Intriguingly, one side effect of CTS-based drugs is psychosis [43] that is often associated with decreased NMDA receptor function [44]. Besides that, it has previously been demonstrated that ouabain induces glutamate excitotoxicity [17]. Thus, the decrease in the amount of NMDA receptor caused by CTS may have important physiological consequences.

After characterizing the effect of CTS binding to Na/K-ATPase on NMDA receptor function, we attempted to study the effect of NMDA receptor activation on Na/K-ATPase activity. We showed that NMDA receptor activation leads to a dose-dependent inhibition of Na/K-ATPase, which corresponds to previously published data [33, 14]. Using inhibitor analysis, we attempted to establish a possible mechanism of Na/K-ATPase inhibition. These experiments demonstrated that this effect of NMDA is prevented by D-AP5 (NMDA receptor inhibitor) and BAPTA (Ca^{2+} chelator). It has been reported that NMDA receptor activation causes Ca^{2+} to enter the cells, leading to protein kinase C activation [45] and PKC phosphorylates Na/K-ATPase, thereby reduces its activity [46]. Therefore, we conclude that Na/K-ATPase inhibition by NMDA receptor activation depends on an increase in intracellular Ca^{2+} . This probably leads to activation of protein kinase C, which can inhibit Na/K-ATPase through the aforementioned phosphorylation [47]. In this manner, the effect is likely possibly through a number of intermediates rather than through direct protein-protein interaction between Na/K-ATPase and NMDA receptor.

Analyzing the dose-response curves of ouabain inhibition of Na/K-ATPase under the simultaneous action of NMDA and D-AP5, we observed that NMDA causes inhibition of the $\alpha 1$ subunit of Na/K-ATPase while D-AP5 prevents this effect. Why activation of NMDA receptors by high concentrations of NMDA causes inhibition of the $\alpha 1$ subunit of Na/K-ATPase? We assume this allows the $\alpha 3$ subunit in restoring the gradient of Na^+ and K^+ after cell excitation and reduces the role of the $\alpha 1$ subunit in the process. This is perhaps an adaptive response, as it is known that different isoforms of Na/K-ATPase

have different affinity to Na^+ ions [48] which leads to different pump function in neurons [49] and differ in the way they are involved in cell signaling [50].

In summary, we have shown for the first time the existence of a functional complex formed by Na/K-ATPase and NMDA receptor. Moreover, both $\alpha 1$ and $\alpha 3$ subunits of Na/K-ATPase can be involved in this interaction.

Acknowledgments We dedicate this article to the memory of Professor Alexander Boldyrev, who passed away in 2012. Professor Boldyrev worked for more than 40 years in this field and created the Moscow school of Na/K-ATPase research at Moscow State University. We thank Professor Amir Askari for the opportunity to perform a part of this work in his Laboratory. We also thank Professor Anita Aperia for the opportunity to do a part of this work in her Laboratory and for her comments on the manuscript. This work was supported by RFBR grants 12-04-31468 and 14-04-32123, the Russian President Grant for 2010/2011, and NIH HL036573. We also received financial support from internal postdoctoral grant from St. Petersburg State University.

Conflict of Interest The authors declare that they have no conflict of interest.

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