Na^+, K^+-ATPase Activity in Children With Autism Spectrum Disorder: Searching for the Reason(s) of Its Decrease in Blood Cells

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Na^+, K^+-ATPase (NKA) activity, which establishes the sodium and potassium gradient across the cell membrane and is instrumental in the propagation of the nerve impulses, is altered in a number of neurological and neuropsychiatric disorders, including autism spectrum disorders (ASD). In the present work, we examined a wide range of biochemical and cellular parameters in the attempt to understand the reason(s) for the severe decrease in NKA activity in erythrocytes of ASD children that we reported previously. NKA activity in leukocytes was found to be decreased independently from alteration in plasma membrane fluidity. The different subunits were evaluated for gene expression in leukocytes and for protein expression in erythrocytes: small differences in gene expression between ASD and typically developing children were not apparently paralleled by differences in protein expression. Moreover, no gross difference in erythrocyte plasma membrane oxidative modifications was detectable, although oxidative stress in blood samples from ASD children was confirmed by increased expression of NRF2 mRNA. Interestingly, gene expression of some NKA subunits correlated with clinical features. Excess inhibitory metals or ouabain-like activities, which might account for NKA activity decrease, were ruled out. Plasma membrane cholesterol, but not phosphatidylcholine and phosphatidlserine, was slighty decreased in erythrocytes from ASD children. Although no compelling results were obtained, our data suggest that alteration in the erytrocyte lipid moiety or subtle oxidative modifications in NKA structure are likely candidates for the observed decrease in NKA activity. These findings are discussed in the light of the relevance of NKA in ASD. ***Autism Research*** *2018, 11: 1388–1403.* © 2018 The Authors. Autism Research published by International Society for Autism Research and Wiley Periodicals, Inc.

Lay Summary: The activity of the cell membrane enzyme NKA, which is instrumental in the propagation of the nerve impulses, is severely decreased in erythrocytes from ASD children and in other brain disorders, yet no explanation has been provided for this observation. We strived to find a biological/biochemical cause of such alteration, but most queries went unsolved because of the complexity of NKA regulation. As NKA activity is altered in many brain disorders, we stress the relevance of studies aimed at understanding its regulation in ASD.

Keywords: autism spectrum disorders; Na^+, K^+-ATPase; erythrocyte membrane; oxidative stress; beta-actin; NRF2; metals; endogenous ouabain; membrane lipids

Introduction

Na+, K + -ATPase (NKA) activity is reduced in a number of brain pathological disorders, including ischemia, injury, depression and mood disorders, mania, stress, neuronal hyperexcitability, and epilepsy [reviewed by de Lores Arnaiz & Ordieres, 2014] as well as in autism spectrum disorders (ASD) [Ghezzo et al., 2013]. These observations prompted us to follow up our previous study on ASD to investigate the origin and possibly the relevance of NKA activity decrease in ASD.

Na^+, K^+-ATPase Structure and Function

NKA is a protein complex localized at the cell membrane, first described by Skou [1957], who identified it as the main factor responsible for the establishment of the sodium and potassium gradient across the cell

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membrane. For each ATP-driven transport cycle, two K^+ ions are imported into the cell while three Na^+ ions are exported from it. NKA activity allows molecules to pass through the cell membrane via indirect active transport, contributes to establish the membrane electrical potential and is fundamental in the propagation of nerve impulses. NKA belongs to the P-type ATPases, a family of enzymes comprising more than 50 members and characterized by having a phosphorylated (P) enzyme intermediate in common. NKA consists of α and β subunits, both required for enzyme function, whereas a third subunit, referred to as FXYD, appears to be involved in regulating enzyme activity in a tissue-specific way. The α subunit is referred to as the catalytic subunit, as it includes the binding sites for ATP and for ion occlusion, as well as for the inhibitor ouabain. It has a relative molecular mass of 100–113 kDa, according to the different isoforms: α1, α2, α3, or α4, and crosses the membrane 10 times, forming transmembrane domains M1–M10, whereas both N- and C-termini are localized on the cytosolic side. Mutations in the α subunits are involved in many (often neurological) diseases [Clausen, Hilbers, & Poulsen, 2017]. The β subunit is in direct contact with the α subunit, acting to facilitate the correct positioning and conformational stability of the α subunit within the plasma membrane. Moreover, the β subunit modulates ion affinity and transport, ATP hydrolysis, and the binding of inhibitors to NKA as a whole. The mass of the protein moiety of β subunit is 36–38 kDa, depending on the different isoforms – β1, β2, or β3; however, as it is heavily glycosylated, it has a relative molecular mass of about 60 kDa. The β subunit crosses the membrane only once, with the N-terminus localized on the intracellular side of the membrane. The FXYD subunit is a type I membrane protein, crossing once the plasma membrane, with the N-terminus localized on the extracellular side and characterized by the conserved FXYD motif (FXYD stands for the amino acids phenylalanine, tyrosine, and aspartate), one conserved serine and two conserved glycine residues. In mammals, the FXYD protein family contains seven members, named FXYD 1–7. As the FXYD subunit is not present in all cell types, it appears not to be essential for NKA activity, but rather, serves to modulate ion transport function through molecular interactions with specific enzyme domains [Scheiner-Bobis, 2002]. The expression pattern of the different isoforms of α, β, and FXYD subunits depends on cell type, developmental stage, and specific signals [Sweadner & Rael, 2000; Hoffman, Wickrema, Potapova, Milanick, & Yingst, 2002; Blanco, 2005; Lubarski, Pihakaski-Maunsbach, Karlish, Maunsbach, & Garty, 2005; de Lores Arnaiz & Ordieres, 2014].

NKA Regulatory Roles

Proteins may associate with the cytoplasmic domains of the enzyme, underlying the fact that NKA carries regulatory roles beyond ion transport. In fact, NKA activity is regulated by the so-called cardiotonic steroids (CTS, mainly digoxin and ouabain); in turn, through its binding to a variety of cytoplasmic proteins, NKA is involved in the regulation of a number of signaling processes, which vary in different tissues and may modulate relevant activities such as intracellular Ca^{2+} concentration, changes in gene expression, cell proliferation, survival and apoptosis, cell–cell contacts and communication, synaptic and neural processes (Goldstein et al., 2006; Matchkov & Krivoi, 2016; Reinhard, Tidow, Clausen, & Nissen, 2013; Xie, 2003).

NKA Regulation

CTS are compounds found in a variety of plants and in few animal species that specifically bind to the extracellular domains of NKA, where binding serves to stabilize the enzyme in its E2 conformation, thus inhibiting its ion transport activity. Examples of plant CTS are ouabain, digoxin, digotoxin, whereas marinobufagenin is the most well-known among the compounds isolated in toads. Ouabain-like and digoxin-like compounds have also been found to be endogenously synthesized in mammals at subnanomolar concentrations, but increase in some physiological and pathological conditions, suggesting a role in the modulation of different processes. The interaction of these compounds with NKA activates its binding to Src kinase and resulting signaling cascades [Pierre & Xie, 2006].

Pyridine, urea (Nikezić et al., 1998), some organophosphate compounds [Blasiak, 1995; Jovanović, Vasić, Nikolic, Ćetković, & Nikezić, 2000] and some metals, notably vanadium (North & Post, 1984; Ehrenspeck, 1980), cadmium and mercury [Vasić, Jovanović, Horvat, Momić, & Nikezić, 2002] have been reported to inhibit NKA activity.

The lipid moiety of the membrane where the enzyme is embedded provides an additional source of NKA regulation [Cornelius, Habeck, Kanai, Toyoshima, & Karlish, 2015; Habeck et al., 2015]. In particular, the thickness of the phospholipid bilayer (related to the acyl chain lengths), the presence of optimal cholesterol concentration [Chen et al., 2011], omega-3 abundance, and the percentage of anionic or neutral phospholipids all affect enzyme stability and activity.

In particular tissues, regulation of NKA activity may depend on post-translational modifications of the FXYD subunit [Fuller et al., 2013]. In addition, changes in the cell redox state and hypoxia/reoxygenation events affect NKA activity and may play an important role in pathological and adaptive responses by affecting a variety of redox-sensitive modifications including S-glutathionylation, S-nitrosylation, and redox-sensitive phosphorylation. Thiol modifications are not only protective towards the inactivating action of oxidants, but may also play regulatory roles [Bogdanova, Petrushanko, Hernansanz-Agustín, & Martínez-Ruiz, 2016]. Each NKA subunity type contains cysteine residues, which

are potentially attacked by oxidants and protected by glutathionylation; however, α2, which is mostly expressed in the heart and in the brain astrocytes, is more susceptible than other isoforms to irreversible oxidation [Xie et al., 1995]. Because of the susceptibility of the enzyme to react with oxidants and glutathione, NKA activity was found to be decreased in a number of pathological and physiological circumstances [Bogdanova et al., 2016], including chronic inflammation, which is associated with oxidative stress [Staroń, Mąkosa, & Koter-Michalak, 2012].

ASD and Oxidative Stress

Our research team contributed to the recognition of oxidative stress as a distinctive feature of ASD [Ghezzo et al., 2013; Abruzzo et al., 2015] and described a marked reduction (to about 35% of control values) of NKA activity in erythrocytes from ASD children; a similar decrease in NKA activity in ASD subjects was also reported by Kurup & Kurup, 2003. It should be pointed out that, according to many recent studies, many neuropsychiatric disorders are *de facto* inflammatory disorders, although the particular mechanisms underlying this association are still under debate [Réus et al., 2015; Young et al., 2016]. Undoubtedly, neuroinflammation finds a correlate in oxidative stress [Emiliani, Sedlak, & Sawa, 2014].

The aim of the present work is to elucidate the mechanisms underlying the reduction in NKA activity observed in ASD children, with reference to the role of NKA in neuropsychiatric and neurodegenerative diseases.

Materials and Methods

Ethics Statement

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and approved by the Local Ethical Committee (Azienda USL Bologna, Imola, Ferrara, CE 13062, 23/12/2013; Prot. N.1198/CE).

Subjects

Twenty-two children diagnosed with non-syndromic ASD (17 males and 5 females, aged (mean ± SD) 7.75 ± 1.87 years, age range 5.25–11.08 years) and 21 typically developing children (14 males and 7 females, aged (mean ± SD) 9.44 ± 1.96 years, age range 5.25–11.83 years) were recruited by the Child Neuropsychiatric Unit of the Bellaria Hospital (IRCCS, Bologna). The patients underwent a clinical diagnostic assessment and a comprehensive neurological work up. The diagnostic evaluation performed by two expert child neuropsychiatrists included a clinical observation following DSM-5 criteria (American Psychiatric Association, 2013), a standardized autism assessment by autism diagnostic observation schedule (ADOS) (Lord et al., 1999) and childhood autism rating scale (CARS) (Short & Schopler, 1988), and finally a cognitive functioning through Leiter-R scale (Tsatsanis et al, 2003) for brief non verbal intelligence quotient (IQ) considering our sample language impairment. Any medical and neurological comorbidity was excluded by electroencephalography (recorded both awake and sleeping), cerebral magnetic resonance imaging, standard clinical and neurological examination and neurometabolic and genetic investigations (including 550 band karyotype, and molecular assay for Fragile X and MECP2). For the ASD group, CARS total scores ranged from mild to severe autistic features; developmental scores varied from normal IQ to severe cognitive impairment (Table 1a). Control typically developing (TD) children were recruited in the same local community and did not display any sign of cognitive, learning, and psychiatric involvement, as assessed by two expert child neuropsychiatrists (Table 1b). All subjects were on the typical Mediterranean diet and did not take any medication or dietary supplements in the 4 months preceding the biochemical and clinical evaluations. Dietary habits were assessed by a food questionnaire, according to the guidelines issued by the Emilia-Romagna Health Authority. No ASD child was on a diet free of gluten or casein.

Materials

All chemicals were analytical grade and were purchased from Sigma-Aldrich (St. Louis MO), unless otherwise specified.

Protein Concentration

Protein concentration was determined as described by Bradford [1976], using serum albumin as a standard.

Blood and Urine Samples

Blood samples (~14 mL), obtained from ASD and TD children, were collected in Na_2-EDTA vacutainers. Basal hematological parameters were examined by routine laboratory techniques using 5 mL whole blood. Erythrocyte plasma membrane preparation was carried out using 1.5 mL of whole blood, according to the method described by Matte et al. [2013]. The remaining blood was centrifuged to separate the plasma, which was stocked at –80 °C. The cell suspension was separated by Ficoll density gradient to obtain mononuclear white blood cells (peripheral blood mononuclear cells [PBMC]). Spot urine samples were collected in sterile containers (FORMESA, Italy). Proteinuria and creatinine determinations were evaluated using standard laboratory techniques. The remaining urine was stored at –80 °C before the analytical evaluation of metal concentration.

Table 1. a. Demographic and Clinical Features of the ASD Children. b. Demographic Features of the Typically Developing Children

a. Demographic and clinical features of the ASD children

No.	Gender	Age (months)	Onset pattern: 1 (early); 2 (regressive); 3 (mixed)	Brief non verbal IQ[1]	ADOS score[2]	CARS total score	CARS activity level item score	CARS body use (stereotypies) item score	CARS verbal communication item score	CARS non verbal communication item score	CARS total number of items whose score was ≥3
1	m	68	1	48	20	41.5	2.5	3	3	3	11
2	m	94	1	52	21	46	3.5	4	4	4	11
3	f	68	3	71	20	43.5	2.5	3	3.5	3	10
4	m	93	2	45	20	48.5	3	4	3.5	3	12
5	m	82	2	55	19	42	3	3	3	3	9
6	m	115	1	52	19	39.5	3	3	3	3	7
7	m	74	3	68	19	41	2.5	3	3	3	9
8	m	99	1	56	16	35	2.5	2	3	3	3
9	m	85	1	62	22	41	2.5	2	3	3	7
10	f	74	1	47	17	38	2	2.5	3	3	7
11	f	124	3	87	21	33.5	2	2	2.5	2.5	4
12	m	129	1	68	19	39	2	2.5	3	3	7
13	m	78	3	82	17	39	3	2.5	3	3	6
14	m	96	1	67	22	44	2.5	3	3	3	11
15	m	84	1	95	18	39.5	2.5	3	3	3	8
16	m	114	1	45	21	47	3	3.5	3.5	3	13
17	m	103	3	47	16	41.5	4	2	3	3	10
18	m	131	1	96	18	35	2	3	2.5	2.5	3
19	f	64	1	75	17	39.5	2.5	2.5	3.0	3.0	6.0
20	f	64	1	74	17	41.0	2.5	2.5	3.0	3.0	7.0
21	m	98	1	49	20	41	2	3.5	2.5	2	9
22	m	133	3	80	13	34	2	1.5	3	2.5	2

b. Demographic features of the typically developing children

No. progr.	Gender	Age (months)
1	f	140
2	f	74
3	f	97
4	f	93
5	m	142
6	m	127
7	m	116
8	m	142
9	m	135
10	m	63
11	f	125
12	m	115
13	m	115
14	m	125
15	f	102

(Continues)

Table 1. Continued

b. Demographic features of the typically developing children		
No. progr.	Gender	Age (months)
16	f	120
17	m	101
18	m	130
19	m	123
20	m	136
21	m	65

The study was conducted according to the declaration of Helsinki guidelines and approved by Local Ethical Committee. Written consent was obtained from parents as well as from children through pictures and simplified information.

[1] Cognitive level: ≥70, normal; 55–69, mild cognitive impairment; 44–54, moderate cognitive impairment; ≤39, severe cognitive impairment.

[2] ADOS modules 1 or 2 (Total score autism cut off = 12).

NKA Activity Assay in PBMC

Na^+/K^+-activated Mg^{2+}-dependent ATPase activity was determined in PBMC cell membranes, as previously reported [Ghezzo et al., 2013]. The results are expressed in μmol Pi/mg prot/h.

PBMC Plasma Membrane Fluidity

PBMC plasma membrane fluidity was evaluated as previously reported [Ghezzo et al., 2013].

Gene Expression

About $4*10^6$ PBMC from 16 ASD and 17 TD children were lysed in 1 mL Trizol® Reagent (Invitrogen, Milan, Italy). Trizol-extracted RNA was quality controlled and quantified as described by Abruzzo et al. [2013]. Quantitative real-time polymerase chain reaction (RT-PCR) was performed in a BioRad CFX96 real-time thermal cycler using the SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA). Custom-designed primer sequences are reported in Supporting Information of Table S1. Using CFX Manager ™ Software (Bio-Rad Laboratories) and qBaseplus (http://www.biogazelle.com), data were analyzed with the $2^{-\Delta\Delta CT}$ method, taking into account the efficiency of the real-time PCR reaction between 95% and 105%. The stability of housekeeping genes was validated according to the criteria suggested by Vandesompele et al., 2002. RT-PCR data are expressed as means ± confidence interval, where a significance level of 0.05 corresponds to the 95% confidence level.

Western Blot (WB) of NKA Subunits in Erythrocyte Plasma Membranes

Twenty μL of ghost suspension (~55 μg protein) were assessed. Technical details are reported in the Supporting Information section. Specific protein band density was quantified by means of BioRad GelDoc 2000 with reference to the fluorescence of each sample lane, generated by exposing the trihalo compounds in the membrane to UV.

Glutathionylation of Erythrocyte Plasma Membrane Proteins

The protocol described by Hill, Ramana, Cai, Bhatnagar, and Srivastava [2010], was applied with minor modifications. Electrophoresis was performed on precast gradient Mini-PROTEAN TGX gels with 10 μL (~30 μg protein) of ghost suspension, solubilized 1 hr in ice bath in LN buffer (200 mM Tris–HCl, pH 6.8, 5% SDS, 25% glycerol, 0.04% Bromophenol blue, 25 mM N-ethylmaleimide. Membranes were probed overnight at 4 °C with the monoclonal primary mouse antibody Glutathione-D8 (Thermo Scientific, Rockford, IL) diluted 1:100 in 0.1%TBS-Tween, then exposed to secondary antibody and quantified as described for NKA subunits.

Table 2. Inhibitors of NKA Activity

a. Plasma and urine concentration of inhibitory metals

Inhibitory metals	TD	ASD	*P* (Mann–Whitney)
	Plasma concentration (μg/L), mean ± *SD*		
Vanadium	0.38 ± 0.19	0.39 ± 0.31	0.885
Cadmium	0.54 ± 0.14	0.52 ± 0.10	0.559
Mercury	0.55 ± 0.13	0.53 ± 0.11	0.587
	Urine concentration (μg/mg creatinine), mean ±*SD*		
Vanadium	$2.90*E^{-4} \pm 3.89*E^{-4}$	$4.63*E^{-4} \pm 4.78*E^{-4}$	0.204
Cadmium	$7.26*E^{-4} \pm 4.47*E^{-4}$	$1.00*E^{-3} \pm 7.01*E^{-4}$	0.133
Mercury	$6.13*E^{-4} \pm 3.83*E^{-4}$	$5.73*E^{-4} \pm 3.01*E^{-4}$	0.706

b. Plasma concentration of endogenous ouabain

TD			ASD		
Pool	Endogenous ouabain (nM)	Mean ±CV	Pool	Endogenous ouabain (nM)	Mean ±CV
A (4 subjects)	0.263	0.270 ± 18.668	D (3 subjects)	0.292	0.270 ± 17.545
B (4 subjects)	0.212		E (3 subjects)	0.313	
C (3 subjects)	0.335		F (4 subjects)	0.204	

The vanadium signal was quantified at m/z 51 in He mode; cadmium signal at 111 m/z in He and mercury signal at 202 m/z in He. The calibration range was from 0.0025 to 25 μg/kg and limit of quantification (LOQ) for V was 0.001 μg/kg and 0.001 mg/kg for Cd and Hg. High purity deionized water was obtained by Evoqua Water Technologies (Barbsbuttel, DE); nitric acid was from J.T. Baker (Center Valley PA, USA). Plasma Endogenous Ouabain was determined by radioimmuneassay. Plasma was mixed overnight with 1:1 methanol to obtain protein precipitation. Protein-depleted plasma was dried out with a speed vacuum concentrator. Dried samples were diluted in PBS and applied to a C_{18} column (Agilent Bond Elut C18 sample prep, Variant Ca, USA), then eluted with a gradient 0-25% PBS:acetonitrile. Eluted Endogenous Ouabain was dried with a speed vacuum concentrator, then reconstituted in radioimmuneassay buffer; quantification was made with polyclonal rabbit antibody (DBA, Italy). Intra- and inter-assay variability was <10%.

Carbonyl Groups in Erythrocyte Plasma Membrane Proteins

Carbonyl groups were assessed with the OxyBlot™ Protein Oxidation Detection Kit (Millipore, USA & Canada) by using 5 μL of ghost preparation for each sample following the manufacturer's instructions. Protein carbonylation was quantified using actin as the loading control.

Metal Concentrations in Plasma and Urine

Urine and plasma samples (3 mL) were thawed and throughly mixed, then 7.5 mL of nitric acid were added in a screw cap polypropylene 50 mL sample tube (Digi-Tubes SCP Science); samples were then placed in a Digi-Prep system (SCP Science) at 75 °C overnight. After cooling, samples were diluted with 7.5 mL of deionized water (Evoqua Water Technologies, Barbsbuttel, DE), followed by a further 5x dilution with 1 N HCl. Analysis was carried out according to the method described in Galimberti et al. [2016], by using an inductively coupled plasma mass spectrometer (ICP/MS 7700, Agilent Technologies USA) coupled with an ASX-500 CETAC Autosampler (Cetac Technologies, Omaha NE, USA). Metal concentration in urine was normalized by creatinine concentration.

Endogenous Ouabain Evaluation

Endogenous ouabain evaluation was possible only for a subgroup of subjects (TD N = 11; ASD N = 10), whose blood sample was large enough to allow for the recovery of an additional 350–450 μL plasma. As the quantification of endogenous ouabain required a minimum volume of 1.3 mL, three or four samples were randomly pooled in order to be able to analyze three pooled plasma samples from TD children and three from ASD children. The method is fully discussed in Ferrandi et al. [1997] and relies on a radioimmunoassay developed with a custom-made rabbit polyclonal antiouabain antisera. Details are reported in the legend of Table 2.

Cholesterol in Erythrocyte Plasma Membrane

Cholesterol was quantified by means of the Fluorimetric Cholesterol Quantification Kit (Sigma-Aldrich) following the manufacturer's instructions, except that the chloroform:isopropanol:IGEPAL CA-630 extraction step was omitted because the starting material was the ghost preparation. For each sample, 2.5 μL of ghost preparation was used in duplicate. A Perkin-Elmer Victor two plate reader was used (A_{570}) to evaluate total membrane cholesterol concentration, expressed as μg/μg of sample protein.

Phosphatidylserine in Erythrocyte Plasma Membrane

Phosphatidylserine was evaluated according to the method described by Morita et al. [2012]. Lipids were extracted from 30 μL of ghost preparation using the Lipid Extraction Kit (chloroform free) (BioVision Milpitas, CA).

Lipid extracts were resuspended in 50 μL of aqueous solution of 1% Triton X-100 (non-ionic, viscous liquid, Roche). A Perkin-Elmer Victor two plate reader was used (λ_{ex} = 544/λ_{em} = 590 nm). Phosphatidylserine concentration is expressed as μg/μg of sample protein.

Phosphatidylcholine in Erythrocyte Plasma Membrane

Phosphatidylcholine was evaluated fluorimetrically using the Phosphatidylcholine Assay kit (Sigma-Aldrich) according to the manufacturer's instructions. The ghost sample was diluted 1:25 and 2.5 μL/well were used, in duplicate. A Perkin-Elmer Victor two plate reader was used to assess samples (λ_{ex} = 535/λ_{em} = 587 nm). Phosphatidylcholine concentration is expressed as μg/μg of sample protein.

Statistical Analysis

Data were tested for normality using the D'Agostino-Pearson test, following which appropriate parametric tests (Student's t for independent data) or the nonparametric equivalent (Mann–Whitney) were used. Parametric correlation (Pearson) was used to correlate clinical features and NKA gene expression data in the ASD group. Differences were considered significant at $P < 0.05$. To account for multiple testing we used the Benjamini and Hochberg false discovery rate (FDR). FDR corrected *P*-values (pFDR) were evaluated separately for (a) comparisons of biochemical parameters in ASD and TD, (b) correlations of clinical features and NKA gene expression data in ASD group, and (c) correlations of NKA gene expression and NRF2 expression in ASD group. RT-PCR data are expressed as means ± confidence interval, where a significance level of 0.05 corresponds to the 95% confidence level.

Results

Subjects

The age range of the two groups studied, TD and ASD children, was the same, but the individual ages were not matched, due to difficulties in sampling control subjects. For this reason, all results were evaluated for possible age-dependence. For both groups of subjects, none of the results obtained in the present study were correlated with age.

NKA Activity and Plasma Membrane Fluidity in PBMC

NKA activity in PBMC from ASD children was significantly decreased in comparison with that of TD children (Fig. 1a). Values (mean ±*SD*) were 1.204 ±0.314 μmol Pi/mg prot/h for TD and 0.661 ±0.273 for ASD children, $P = 0.0002$ by Mann–Whitney, pFDR = 0.0048. NKA activity was not correlated with clinical features.

The decrease of NKA activity in PBMC of ASD children was not associated with differences in PBMC plasma membrane fluidity (Fig. 1b), at variance with previous results found for erythrocytes, where a marked decrease in NKA activity was accompanied by a significant decrease in membrane fluidity [Ghezzo et al., 2013].

Gene Expression in PBMC

Gene expression of isoforms α1, α2, α3, β1, β2, β3, FXYD2, FYXD5, and of Nuclear factor (erythroid-derived 2)-like 2 (NRF2) was evaluated in PBMC from 16 ASD and 17 TD children. The expression of isoform α2 was below the detection level; among the detectable isoforms, β2 was the least expressed. PBMC from ASD children were found to express higher levels of mRNA for the isoforms α1 (+19%) and β3 (+31%) and lower levels for FXYD5 (–18%) than PBMC from TD children (Fig. 1c). NRF2 mRNA expression was found to be 1.56 times greater in ASD than TD children (95% CI 0.953–2.418, $P < 0.05$). Significance was lost after correction for multiple measurements.

Protein Expression of NKA Subunits and Actin in Erythrocyte Membranes

NKA subunit protein expression was evaluated by WB using UV-exposed TGX gels fluorescence, proportional to the protein loaded in each lane, as the loading control. For all proteins examined, representative pictures of gels, nitrocellulose membranes, and target-specific bands are shown in Figure. S1 a–f. Figure 1d shows the histograms representing target protein to total lane fluorescence ratio in the nitrocellulose membrane (mean ±*SD*).

Beta-actin is the most widely used loading control for Western Blotting, however, we chose TGX gel fluorescence based on the observation reported by Cortelazzo et al. [2014], who found that erythrocytes from children affected by Rett syndrome (a monogenic disease where subjects display autistic features) had decreased β-actin content, as compared to healthy children. However, our results showed no difference in β-actin content between TD and ASD children (Fig. 2a–c). This result allowed us to use β-actin as loading control when assessing protein carbonylation (see below).

Both the α and β subunits of erythrocyte NKA were difficult to detect, as already stressed by Arystarkhova and Sweadner [1997]. In particular, α1 and α3 subunits, expected to appear as bands at MW 112 and 110 kDa, respectively, stained at approximately MW 95 kDa (Fig. S1a and b). For this reason, a pan-alpha antibody was additionally used in the attempt to improve the detection of α subunits. As shown in Figure S1c, such antibody stained the same band at apparent MW 95 kDa, as expected according to the antibody manufacturer; however, the band was as faint as those detected by antibodies directed against the α1 and α3 subunits.

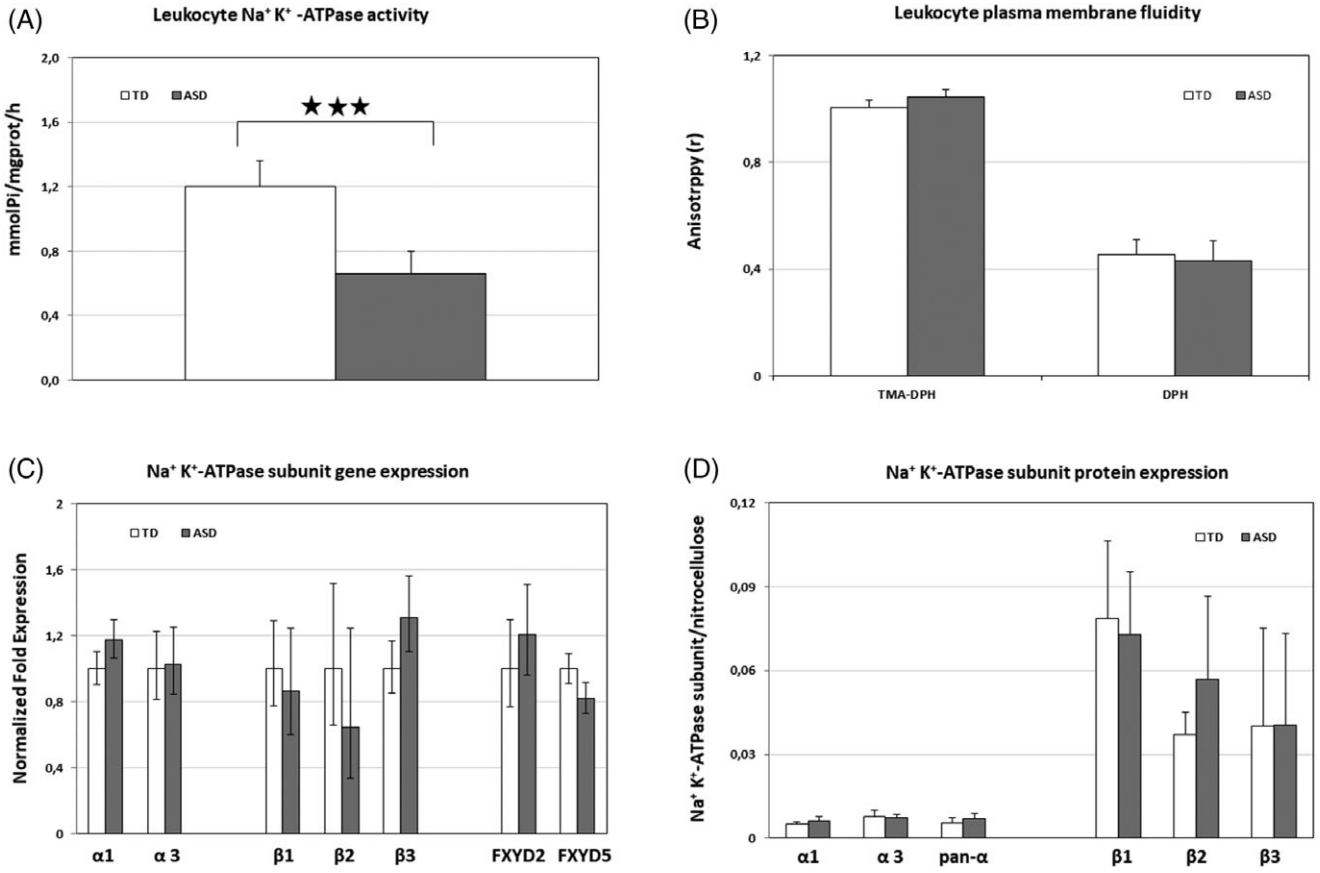

Figure 1. NKA activity, membrane fluidity, gene expression of NKA subunits in PBMC, and protein expression of NKA subunits in erythrocytes from TD and ASD children. A: NKA activity in PBMC from TD and ASD children. B: Plasma membrane fluidity of PBMC from TD and ASD children. The fluorescence anisotropy of the probes TMA-DPH and DPH, which localize in the outer and the inner leaflet of cell membrane, respectively, is inversely related to the fluidity of the microenvironment in which the probes are located. C: Gene expression of NKA subunits in PBMC from TD and ASD children. For each subunit mRNA, expression level is set to 1 for TD mRNA. Histograms show the mean ± confidence. Primer details are reported in Supporting Information of Table S1. D: Protein expression of NKA subunits in plasma membrane erythrocytes from TD and ASD children. Histograms show the target protein/total lane fluorescence ratio in the nitrocellulose membrane (mean ± *SD*); 3 stars = $P < 0.001$.

Moreover, a number of aspecific bands were consistently stained at different MWs.

The datasheet for the β1 antibody shows a single band around 42 kDa for liver and a band around 55 kDa for kidney; the datasheet for the β2 antibody shows a single band at 37.84 kDa; the datasheet for the β3 antibody shows a smear between 40 and 60 kDa. However, in our assays, all β subunits displayed multiple bands in the MW range of 34–60 kDa, probably owing to different levels of glycosylation, and appeared as rather faint bands (Fig. S1d–f). The optical densities of these multiple bands were summed for the evaluation of protein expression. Noteworthy, although we used the same primary antibodies used by Hoffman et al. [2002], when commercially available, our WB patterns differed from theirs, probably owing to the fact that we did not extract bulk purified NKA proteins, but rather, compared the protein expression of single subunits in children erythrocytes.

An attempt to quantify FXYD2 was carried out, but the very low MW of the protein (7 kDa), together with its low amount, did not allow for a reliable quantification by WB; FXYD5 is expected to have a very low expression in erythrocytes (http://biogps.org/#goto=genereport&id=53827).

With the above-mentioned caveats, our data appear to suggest that the protein expression level of α3 subunits and of β1, β2, and β3 subunits of NKA did not differ between erythrocytes from TD and ASD children, whereas α1 appeared to be 1.2-fold increase in ASD samples (the difference was not statistically significant after correction for multiple measurements) (Fig. 1d).

Oxidative Modifications of Plasma Membrane Proteins

The evaluation of glutathionylation and carbonylation of plasma proteins, two relevant protein modifications due to oxidative stress, was carried out on whole extracts

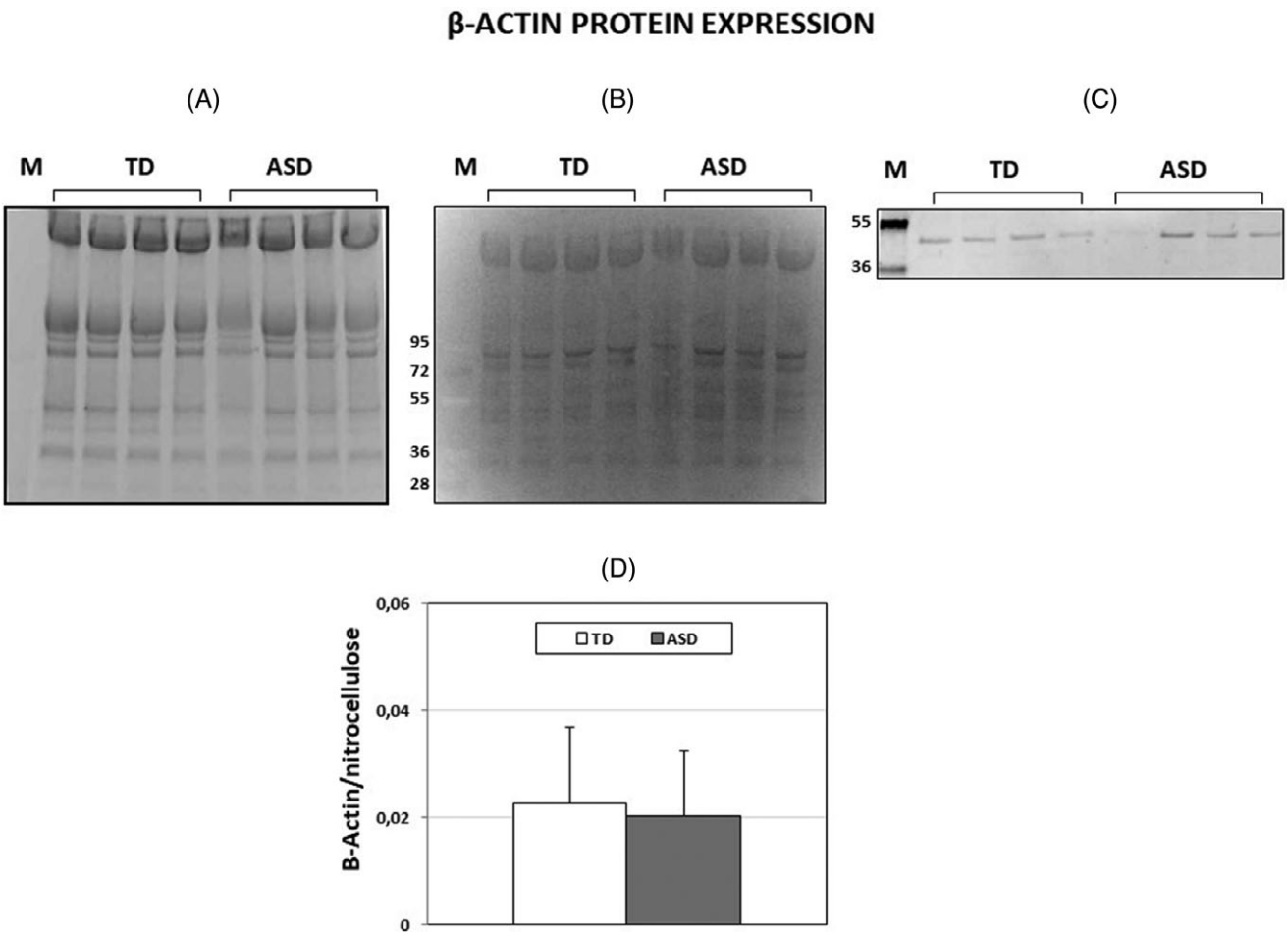

Figure 2. Beta-actin protein expression in erythrocytes from TD and ASD children. A: A representative SDS-Gel electrophoresis of ghost samples. Precast Mini-PROTEAN TGX stain-free protein gel, 4–15% polyacrylamide gels were used. B: Nitrocellulose membrane blotted from the gel of Figure 2A. The fluorescence produced under UV light by trihalo compounds is proportional to the total protein. Samples from TD erythrocytes were run in **lanes 1–4**, samples from ASD erythrocytes were run in **lanes 5–8**. **Lane M**: M.W. markers. C: Beta-actin bands developed from the membrane by an anti-actin antibody marked with Cy5 (see Supporting Information of Table S2). D: Histogram showing means ± *SD*. of β-actin/total lane fluorescence ratio.

of erythrocyte membrane proteins. Glutathione-protein complexes were quantified relative to the lane fluorescence in nitrocellulose membranes (Fig. 3a). Protein carbonyl groups were quantified relative to the actin band density (Fig. 3b). No difference in the amount of glutathione-protein complexes and in carbonylated protein residues was found in erythrocyte membranes of TD and ASD children. Representative gels are shown in Figure S2a and b.

Metal Concentration in Plasma and Urine

We evaluated the plasma and urine concentration of vanadium, cadmium, and mercury, which are thought to inhibit NKA activity [North & Post, 1984; Ehrenspeck, 1980; Vasić et al., 2002]. No difference was found in their concentration between TD and ASD children (Table 2a).

Endogenous Ouabain Evaluation

The analysis of the three pooled plasma samples from ASD and from TD children yielded the same mean and coefficient of variation for the two groups. Data are reported in Table 2b.

Lipids in Erythrocyte Membranes

Although differences in the composition of erythrocyte membrane fatty acids between ASD and TD children was reported previously [Ghezzo et al., 2013], differences in cholesterol, phosphatidylserine, and phosphatidylcholine were assessed in the present work; the choice of lipids is related to their regulatory role on NKA activity [Cornelius et al., 2015]. Data, shown in Figure 4a–c, demonstrate that cholesterol is decreased in erythrocyte membranes from ASD children, whereas no difference was found for PS and PC. After

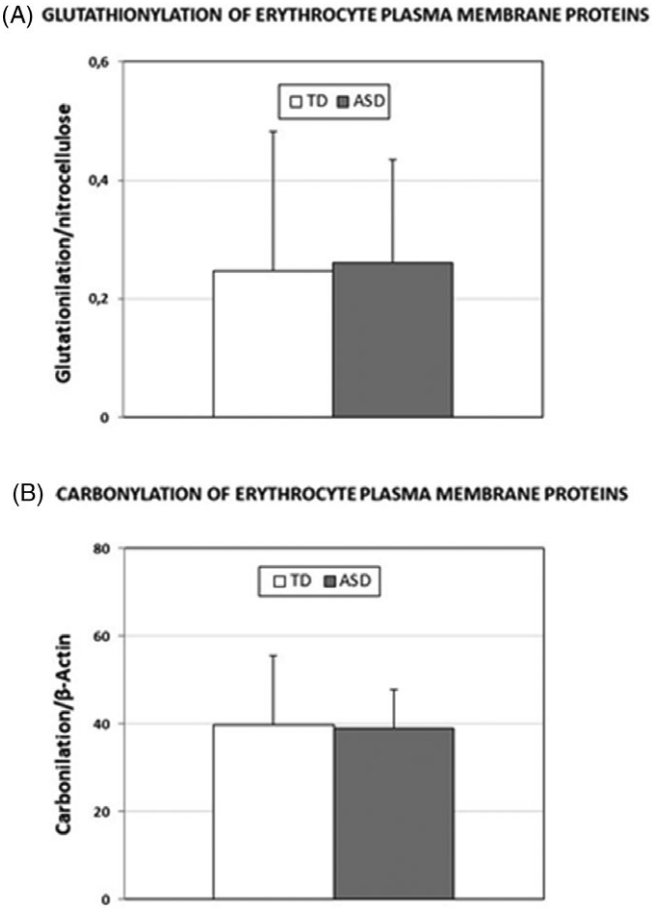

Figure 3. Glutathione-protein complexes and carbonylated protein residues in erythrocyte membranes from TD and ASD children. A: Glutathione-protein complexes in erythrocyte membranes from TD and ASD children were detected by immunoblotting on membranes transferred from TGX gels and quantified relative to the lane fluorescence in nitrocellulose membranes (Means ± *SD*). B: Carbonylated protein residues in erythrocyte membranes from TD and ASD children were immunodetected following their derivatization with DNPH and quantified relative to the actin band density (Means ± *SD*).

correction for multiple measurements, the difference in cholesterol concentration lost statistical significance.

Statistics and Correlation with Clinical Data

Table 3 reports the correlations between NKA subunit or NRF2 gene expression and clinical features. Alpha and Beta NKA subunit gene expression correlated with CARS total scores and with some clinical features. Correlations of NRF2 gene expression and the expression of some NKA subunits are shown in Figure 5.

Discussion

Non-syndromic autism is a complex neurodevelopmental disorder resulting from a combination of genetic and environmental factors [Lai, Lombardo, & Baron-Cohen, 2014]. No peripheral ASD biomarkers have thus far been validated [Abruzzo et al., 2015], however, erythrocyte NKA activity appears very promising in terms of specificity and sensitivity providing motivation for the present study seeking to elucidate mechanism(s) responsible for the marked reduction in NKA activity in erythrocytes of ASD children and to determine, ultimately, whether this plays a role in the etiopathology of non-syndromic ASD.

Although our results extended to PBMC confirm the marked reduction in NKA activity previously reported for erythrocytes [Ghezzo et al., 2013], understanding the underlying mechanisms proved very demanding and elusive. Gene expression of NKA subunits expressed in PBMC was slightly unbalanced in favor of the α1 and the β3 isoforms in ASD and of the FXYD-5 isoform in TD subjects. However, the assessment of protein expression of the NKA subunits, estimated in erythrocyte membranes, did not convincingly support the gene expression data, either because of differences between erythrocyte and PBMC isoform preferences [Clausen et al., 2017] or perhaps owing to the poor performance of commercial antibodies. On the other hand, gene expression values of NKA subunits were correlated with clinical data, thus suggesting that the unbalance in the ASD group was not incidental.

The choice of expressed NKA subunits may be important for tissue function [Matchkov & Krivoi, 2016]. In fact, the individual α, β, and FXYD isoforms are characterized not only by different tissue expression but also by different kinetic/functional properties and by the ability to interact with tissue-specific proteins. For example, the α3 isoform is more expressed in neurons and the α2 isoform in glial cells; neurons mainly display β1 and β2 isoforms, whereas astrocytes, oligodendrocytes, and retinal photoreceptors preferentially express β3. Moreover, development-dependent shifts in isoform expression have been reported: for instance, developing rat brain cells express more of the α2 isoform whereas adult rat brain cells express more of the α3 isoform [Blanco, 2005]. In addition, changes in isoform expression, as well as in ouabain sensitivity, have been reported to occur in the aging brain of the rat [reviewed in de Lores Arnaiz & Ordieres, 2014]. Likewise, the above-described differences in isoform preferences found in PBMC may play a role in NKA activity but may or may not reflect similar differences in brain cells that would affect their function.

Next, we concentrated on the possibility that NKA activity was impaired by oxidative stress, which is now recognized as a distinctive trait of ASD [reviewed in Rossignol & Frye, 2014]. To support the evidence of oxidative stress, gene expression of NRF2, a transcription factor triggered by oxidative stress and inducing antioxidant proteins, was evaluated; although it increased 1.56-fold

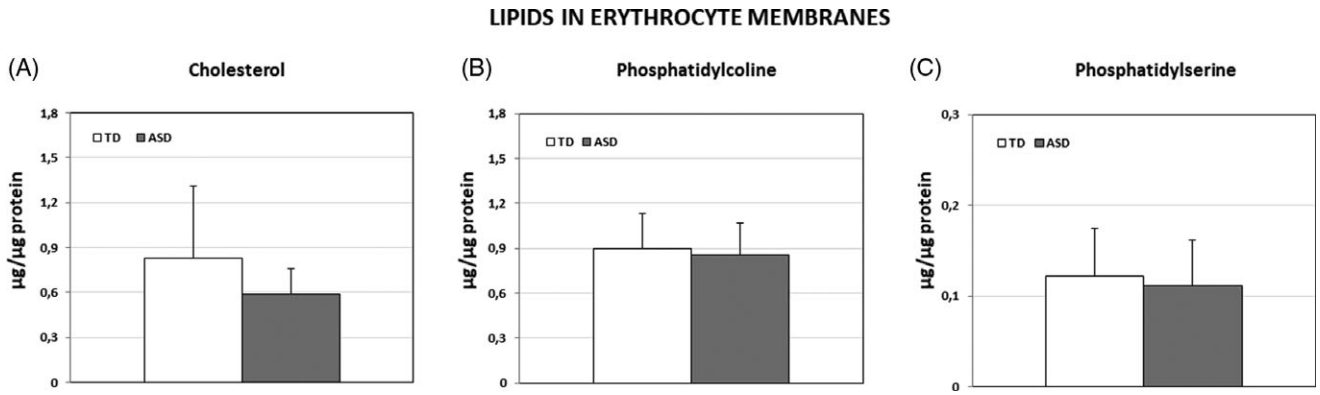

Figure 4. Concentration of selected lipids in erythrocyte membranes from TD and ASD children (µg /µg of sample protein). A: Cholesterol (mean ± *SD*). B: Phosphatidylcholine (mean ± *SD*). C: Phosphatidylserine (mean ± *SD*).

in the PBMC of the ASD children examined here, the difference lost significance after FDR analysis. Whereas all NKA subunits are potentially subject to [inhibitory] oxidative modifications, the subunit more susceptible to irreversible oxidation [Xie et al., 1995] is the α2 isoform, which is richer in cysteine residues but is not expressed in blood cells. Although we did not observe an increase in oxidative modifications of ASD erythrocytes' membrane proteins, subunit-specific differences might have gone undetected due to the fact that we assessed whole extracts of the membrane. However, we decided against enriching our extracts by immunoprecipitation of NKA subunits, due to the low amount of each subunit and a lack of confidence in the specificity of the commercially available antibodies.

Many investigations of the environmental concauses of ASD have reported an increase of toxic metals in biological tissues and fluids of ASD patients [reviewed by Kalkbrenner, Schmidt, & Penlesky, 2014; Rossignol, Genuis, & Frye, 2014], and attention was drawn in particular to heavy metals such as mercury, lead and cadmium, whereas vanadium was not mentioned. As vanadium, mercury, and cadmium are known inhibitors of NKA activity [North & Post, 1984; Ehrenspeck, 1980; Vasić et al., 2002], we evaluated the concentration of these metals in plasma and urine samples. In particular, 3 mL of plasma were used to increase the reliability of the detection, but no differences were found between TD and ASD samples.

Table 3. Gene Expression Fold Change and Correlations With Clinical Features

	Fold change (ratio ASD/TD)	CARS total score	CARS activity level item score	CARS body use (stereotypies) item score	Brief non-verbal IQ
NKA Alpha 1 subunit	1.18	***P* = 0.0004** **R = 0.78** **pFDR = 0.0042**	***P* = 0.0076** **R = 0.63** **pFDR = 0.02**	***P* = 0.0038** **R = 0.68** **pFDR = 0.02**	*P* = 0.031 R = 0.54 pFDR = 0.07
NKA Alpha 3 subunit	1.03	***P* = 0.0013** **R = 0.73** **pFDR = 0.010**	***P* = 0.011** **R = 0.62** **pFDR = 0.03**	*P* = 0.045 R = 0.50 pFDR = 0.08	***P* = 0.0061** **R = 0.68** **pFDR = 0.02**
NKA Beta 1 subunit	0.86	***P* < 0.0001** **R = 0.84** **pFDR = 0.0032**	***P* = 0.0003** **R = 0.79** **pFDR = 0.0048**	***P* = 0.0040** **R = 0.68** **pFDR = 0.018**	*P* = 0.07 R = 0.45
NKA Beta 2 subunit	0.65	***P* = 0.0063** **R = 0.65** **pFDR = 0.02**	***P* = 0.0029** **R = 0.69** **pFDR = 0.018**	*P* = 0.037 R = 0.50 pFDR = 0.07	*P* = 0.09 R = 0.42
NKA Beta 3 subunit	1.31	*P* = 0.024 R = 0.56 pFDR = 0.06	*P* = 0.163 R = 0.36	*P* = 0.141 R = 0.38	*P* = 0.025 R = 0.55 pFDR = 0.06
NKA FXYD2 subunit	1.21	*P* = 0.22 R = 0.32	*P* = 0.144 R = 0.38	*P* = 0.62 R = 0.13	*P* = 0.81 R = 0.03
NKA FXYD5 subunit	0.82	*P* = 0.079 R = 0.45	*P* = 0.131 R = 0.39	*P* = 0.31 R = 0.26	*P* = 0.11 R = 0.41
NRF2	1.56	*P* = 0.022 R = 0.56 pFDR = 0.06	*P* = 0.044 R = 0.51 pFDR = 0.09	*P* = 0.72 R = 0.09	*P* = 0.38 R = 0.23

Statistically significant values are shown in bold.

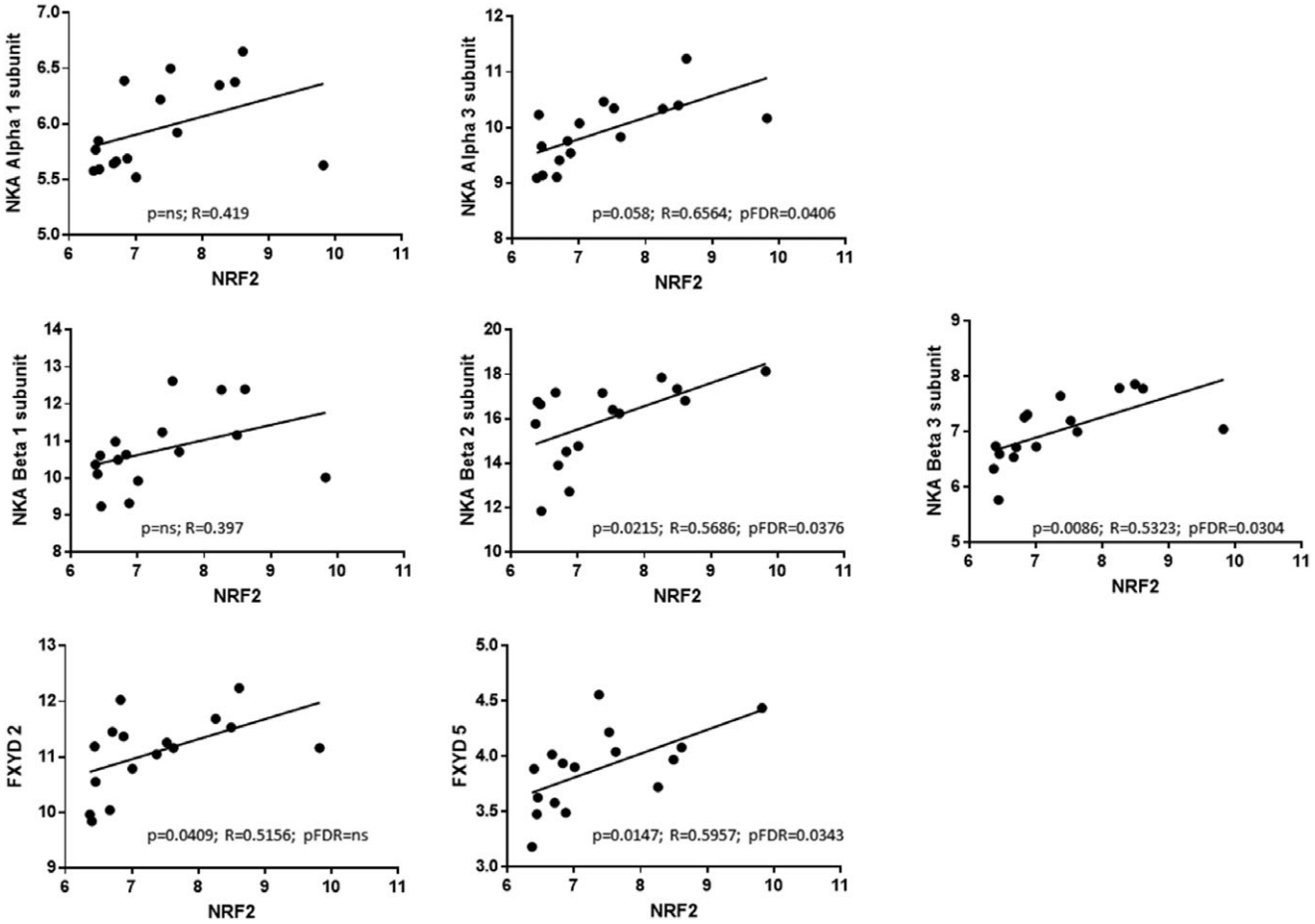

Figure 5. Correlation between gene expression of NRF2 and NKA subunits. pFDR = Benjamini–Hochberg FDR corrected *P* values.

In humans, all NKA isozymes are sensitive to ouabain, whereas the sensitivity to digoxin is more isozyme-selective. In addition, it has been shown that ouabain and digoxin or digitoxin can have opposite effects on blood pressure [Manunta, Hamilton, Rogowski, Hamilton, & Hamlyn, 2000; Zulian et al., 2013]. Endogenous ouabain levels in blood plasma had never been evaluated in ASD patients. Our results showed no difference in the plasma levels of this potent inhibitor of NKA activity between TD and ASD subjects.

We observed a decrease of plasma membrane cholesterol in the erythrocytes from ASD children, thus confirming a previous finding by Schengrund, Ali-Rahmani, and Ramer [2012], who also observed a decrease in monosialotetrahexosylganglioside (GM1) content, and no difference in phosphatidylcholine and phosphatidylserine content. Petrov, Kasimov, and Zefirov [2017] recently pointed out that cholesterol dysfunctions have been detected in many neurodegenerative disorders, however neither Schengrund' or Petrov' groups related these findings to NKA activity. Membrane lipid moiety plays a very important regulatory role on NKA, as pointed out by Cornelius et al. [2015] and Habeck et al. [2015], who in particular draw attention to cholesterol and phosphatidylserine for their stabilization roles and to sphingomyelin and phosphatidylcholine for their inhibitory functions. In particular, cholesterol specifically binds to three different NKA lipid-binding sites. Omega-3 lipids, and notably DHA, tend to inhibit NKA activity, especially in cholesterol-poor membranes. Noteworthy, Cornelius et al. stressed that, because cholesterol does not mix well with DHA, the effect of the two lipids on the conformation of NKA is the opposite; cholesterol increases the lateral pressure in the middle of the bilayer and decreases it near the interfaces, whereas DHA has the opposite effects. Because erythrocyte membranes from ASD patients show depleted DHA [Ghezzo et al., 2013; Giacometti et al., 2017], the effect of a relative depletion in *both* cholesterol and DHA could have on NKA activity may be worth examining in a future work. Likewise, contrary to what we have observed, in kidney cells a decrease in membrane cholesterol was reported to cause a *reduction* in α1 expression and viceversa [Chen et al., 2011]. NAK activity is impaired in both PBMC and erythrocytes, yet we showed that only erythrocytes display a decrease in membrane fluidity. In order to help to resolve these seemingly discrepant results, we suggest that future

studies compare the lipid composition of TD and ASD PBMC membranes .

The observed reduction in NKA activity in erythrocytes from ASD patients apparently does not impact some important cellular functions; in particular, no impairment in immune functions or in oxygen transport or delivery has been reported. However, blood cells may be just the peripheral spyhole on events occurring in the central nervous system, where the relevance of NKA activity cannot be underestimated. NKA plays a crucial role in the maintenance and propagation of the action potential, controls glutamate release in spinal cord [Li & Stys, 2001], mediates glutamate transporter activity [Rose et al., 2009], affects dopamine receptor densities [Hazelwood, Free, Cabrera, Skinbjerg, & Sibley, 2008], regulates ionotropic glutamatergic AMPA receptor turnover [Zhang et al., 2009], interacts with δ-opioid receptor [Deng et al., 2009] and with adenosine A2A receptors controlling glutamate uptake in astrocytes [Matos, Augusto, Agostinho, Cunha, & Chen, 2013]. Findings of decreased brain NKA activity in aging and in many pathological conditions have been reviewed by de Lores Arnaiz et al., (2014), who pointed out that there may be as many mechanisms leading to NKA activity impairment as there are pathological conditions where NKA activity is depressed. Taken as a whole, these findings underscore the relevance of the impairment of NKA activity in neuropsychiatric disorders.

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Declaration

The authors declare the absence of conflicts of interest.

List of abbreviations

ASD	autism spectrum disorders
CTS	cardiotonic steroids
MW	molecular weight
NKA	Na^+, K^+-ATPase
PBMC	peripheral blood mononuclear cells
NRF2	nuclear factor (erythroid-derived 2)-like 2
RT-PCR	real-time polymerase chain reaction
TD	typically developing
WB	western blot
DSM-5	diagnostic and statistical manual of mental disorders-fifth edition
ADOS	autism diagnostic observation schedule
CARS	childhood autism rating scale
IQ	intelligence quotient
MECP2	methyl-CpG binding protein 2

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1: Primer sequences and amplicon length of the genes studied by qRT-PCR

Table S2: Primary and secondary antibodies used in WBs

Figure S1. NKA subunit expression in erythrocyte membranes. Representative pictures of gels, nitrocellulose membranes and Western Blots. TD erythrocytes: lanes 1–4; ASD erythrocytes: lanes 5–8. M: MW markers. a**:** Anti-alpha 1 subunit antibody; b**:** Anti-alpha 3 subunit antibody; c**:** Anti-pan alpha subunit antibody; d**:** Anti-beta 1 subunit antibody; e**:** Anti-beta 2 subunit antibody; f**:** Anti-beta 3 subunit antibody. Details of antibodies are reported in Table S2 and histograms in Figure 1D. Ghost suspensions were solubilized 1 hr in ice bath in Laemmli buffer 4x. Precast gradient gels (Mini-PROTEAN TGX Stain-Free Protein Gel, 4–15% polyacrylamide, Bio-Rad Laboratories, Hercules, CA) and Bio-Rad nitrocellulose membranes were used. After blocking in Tris-Buffered Saline containing 0.05% Tween-20 (TBS-T) and 1% BSA for 1 h at room temperature, membranes were probed overnight at 4 °C with primary antibodies, washed three times with TBS-T and incubated with Cy5-conjugated secondary antibodies, dissolved in TBS-Tween and 0.2% BSA. TGX gels contain trihalo compounds, which, under UV-light, react with tryptophan residues producing fluorescence, proportional to the total protein amount.

Although the ratio gel-to-membrane fluorescence did not substantially differ from one lane to the next, the membrane fluorescence was considered to be more representative and used as loading control.

Figure S2. a**:** A representative gel and its nitrocellulose membrane of glutathione-protein complexes in erythrocyte membranes from TD and ASD children. TGX gels were exposed to UV light and then electroblotted. Membranes were probed overnight at 4 °C with the monoclonal primary mouse antibody Glutathione-D8 (Thermo Scientific, Rockford, IL) diluted 1:100 in 0.1% TBS-Tween, then exposed to secondary antibody. The lane fluorescence was used for quantification of the glutathione-protein complexes (Fig. 2a). Samples from TD erythrocytes were run in lanes 1–4, samples from ASD erythrocytes were run in lanes 5–8. Lane M: MW markers. b**:** A representative gel reacted with anti-DPNH antibody to detect carbonylated protein residues in erythrocyte membranes from TD and ASD children. Each sample was DNPH-derivatized (D) (lanes 1, 3, 5, and 7) or not derivatized (ND)(lanes 2,4, 6, and 8). Lanes 1–4: samples from TD erythrocytes; lanes 5–8: samples from ASD erythrocytes; lane DM: DPNH-derivatized MW markers. On the right, a detail showing the β-actin bands, where HPRT-conjugated secondary antibody was used (See Table S2). Protein carbonyl groups in DNPH-derivatized lanes were quantified relative to their actin band density (Fig. 2b).