Comparative analysis of alternating hemiplegia of childhood and rapid-onset dystonia-parkinsonism ATP1A3 mutations reveals functional deficits, which do not correlate with disease severity

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1. Introduction

The P-type Na⁺/K⁺-ATPases are integral membrane proteins that actively transport three sodium ions (Na⁺) out of the cell and two potassium ions (K⁺) into the cell, using the energy of ATP hydrolysis. Thereby, they establish and maintain an electrochemical gradient at the plasma membrane, which is essential for diverse cellular functions including neuronal activity (Albers, 1967; Post et al., 1972; Skou and Esmann, 1992). Na⁺/K⁺-ATPases are composed of a catalytic α-subunit, and are typically also interacting with a small, regulatory subunit of the FXYD family. The β-subunit is known to be important for the correct folding and membrane localization of the α-subunit. Both the β- and the FXYD (γ-) subunits have been shown to modulate Na⁺/K⁺-ATPase activity (Cornelius and Mahmood, 2003; Geering, 2008). In humans, four different α isoforms (α1 - α4) with different tissue- and development-dependent expression patterns are known (Blanco, 2005; Blanco and Mercer, 1998). ATP1A3 encodes the α3 isoform of the Na⁺/K⁺-ATPase, which in the central nervous system is exclusively expressed in neurons (Bottger et al., 2011; Dobretsov and Stimers, 2005; McGrail et al., 1991). Mutations in ATP1A3 have been linked to various neurological disorders, including rapid-onset dystonia-parkinsonism (RDP) and alternating hemiplegia of childhood.
(AHC). RDP is a rare dystonic movement disorder characterized by a slow or an abrupt onset of dystonia with features of parkinsonism that can last from a few minutes to one month in combination with prominent bulbar findings (de Carvalho Aguiar et al., 2004; Haq et al., 2019). AHC is characterized by early-onset episodes of hemiplegia or hemidystonia, various paroxysmal symptoms and developmental impairment (Heinzen et al., 2012; Rosewich et al., 2012). While most documented mutations are associated with either RDP or AHC, in some cases the same pathogenic variant was reported to lead to different clinical outcome (e.g. D923N, (Roubergue et al., 2013b)), leading to the hypothesis that both diseases constitute a clinical continuum with AHC at the severe end and RDP as a milder variant (Rosewich et al., 2014). Although AHC phenotype is often more severe compared to RDP, some uncertainty exists regarding the severity of the phenotype for the different mutations. For some mutations there is only a handful of documented cases. In other cases, particularly familial RDP, wide spectrum of severity is associated with the same mutation.

The mechanisms that determine the severity of AHC and RDP symptoms are far from being fully understood. Many AHC mutations are clustered close to the ion binding sites (Holm et al., 2016; Kanai symptoms are far from being fully understood. Many AHC mutations of severity is associated with the same mutation. Weiss et al., 2014). Enzymatic studies demonstrated reduction in a emphasize the pump function is still limited. Both AHC and RDP mutations impair the pump function is still limited. Both AHC and RDP mutations demonstrate reduced ATPase activity (Heinzen et al., 2012; Weigand et al., 2014). Enzymatic studies demonstrated reduction in Na2-affinity for some RDP mutations (Blanco-Arias et al., 2009; Einholm et al., 2010; Holm et al., 2015; Pedersen et al., 1997; Poulsen et al., 2010; Rodacker et al., 2006; Toustrup-Jensen et al., 2014). For some AHC mutations, loss of Na2-activated phosphorylation has been reported, as well as no ouabain binding (Weigand et al., 2014), reduced pump current, dominant-negative effects, and loss of proton current (Li et al., 2015). Misfolding of the α subunit for some mutations, and competition between mutant and normal alleles on β subunits have recently been proposed as additional factors which may contribute to the phenotypic outcome (Arystarkhova et al., 2019).

The aim of this study was to compare mutations from both, RDP and AHC, for their effects on protein expression, cell survival and pump current, as potential determinants of disease severity. None of these measures showed clear distinction between the phenotypes. For both phenotypes, high protein expression was measured, with some variation between mutations. All mutations demonstrated functional impairment as reflected by significantly lower cell survival under ouabain challenge, and most of the mutations led to a significant reduction in the pump current, with I274N and I274T showing the most prominent effect and I758S showing no reduction in current.

2. Material and methods

2.1. Constructs and site-directed mutagenesis

Full-length human ATP1A3 cDNA clone in the expression vector pcMV6-XL5 was obtained from OriGene (OriGene Technologies, Inc.) and used as a template for site-directed mutagenesis. To obtain an ouabain resistant construct, we introduced the mutations Q108R (c.323A > G) and N119D (c.355A > G) (Jewell and Lingrel, 1991; Price et al., 1990). We produced twelve mutant constructs by introducing point mutations into the ouabain resistant construct: I274T (c.821T > C), F780L (c.2338T > C), D801Y (c.2401G > T), V322D (c.965T > A), D801N (c.2401G > A), E815K (c.2443G > A), I274N (c.821T > A), D923Y (c.2767G > A), D923N (c.2767G > A), G947R (c.2839G > A), T613M (c.1838C > T) and I758S (c.2273T > G). All constructs were amplified using Pfu polymerase (Promega) and ligated into vector pCDNA3.1TM-myc-His (−)B (Invitrogen) via Xhol and HindIII sites. For expression in Xenopus laevis oocytes, wild type or mutant ATP1A3 constructs were excised from pcDNA3.1TM-myc-His (−)B using restriction enzymes Xhol and HindIII. Resulting fragments were ligated into vector PSEGEM via Xbal and HindIII sites, in which a myc-tag was inserted by oligo-hybridisation using oligonucleotides Oligo_myc for (Hind/Xho) and Oligo_myc rev (Hind/Xho). Full-length human ATP1B1 was inserted into vector PSEGEM containing myc tag via Xbal and EcoRI. All constructs were verified by sequence analysis. Base count was started from the A in the start ATG (GenBank RefSeq accession numbers: ATP1A3 NM_152296.4; ATP1B1 NM_001677.3). The sequences of the oligonucleotides used for plasmid constructions are listed in Table S1.

2.2. Cell culture and transfection

HEK 293FT cells were grown in Dulbecco’s modified Eagle's medium (DMEM) high glucose, supplemented with 10% fetal calf serum, 1% l-glutamine and 1% penicillin-streptomycin at 37 °C and 5% CO2. One day before transfection, cells were seeded in 6-well plates at a density of 3 104/cm2. Cells were transiently transfected using lipofectamine LTX according to the manufacturer's protocol. Transfection efficiency was assessed by fluorescence-activated cell sorting (FACS) analysis (Fig. S1).

2.3. Immunoblotting

Transfected cells were harvested in phosphate-buffered saline (PBS), centrifuged for 10 min (210 g, 4 °C) and the supernatant was discarded. Cell pellet was resuspended in RIPA-buffer containing150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1% (v/v) SDS, 0.5% (v/v) sodium-deoxycholate, 1% (v/v) NP-40 and Complete protease inhibitor cocktail (Roche, catalog No. 04693116001). After 30 min incubation on ice, the cells were centrifuged for 15 min (18,620 g, 4 °C) and the supernatant was collected. Protein concentration was measured in BCA assay (Interchim BC assay kit) and lysate was mixed with Laemmli buffer and incubated for 10 min at 37 °C. 35 μg of protein was loaded in each lane of 8% polyacrylamide gels. SDS-PAGE was performed for 2 h at 15 mA. Blotting was done at room temperature for 75 min at 65 mA in transfer buffer containing: 25 mM Tris-HCl, 160 mM glycine and 20% v/v methanol. The membrane was blocked with 5% nonfat dried milk (Roth, T145.3) in 0.1% Tween 20 in PBS, followed by overnight incubation at 4 °C with primary antibody anti-ATP1A3 (Santa Cruz sc-374050, 1:100) in blocking solution. GAPDH was used as a housekeeping protein (Abcam ab8245, 1:10,000) to correct for loading errors. Incubation with secondary horseradish peroxidase (HRP)-conjugated antibody (donkey anti-mouse, Jackson ImmunoResearch, 1:10,000) was followed by detection with Lumi-Light or Lumi-Light plus (Roche) for GAPDH and ATP1A3, respectively. Signals were detected by ImageQuant LAS 4000 mini and analyzed with ImageJ.

To allow for quantitative evaluation, we ascertained that the amount of loaded protein lies within the linear range (Fig. S2). We used the intensity ratio ATP1A3/GAPDH for the comparative analysis and the intensity of the wild type construct as a reference level for the mutant constructs. All mutants were loaded on a single gel to enable a careful comparison of all band intensities (Fig. 1A).

2.4. Ouabain challenge test

HEK 293FT cells were seeded in 6 well plates at a density of 3 104/cm2 and transfected after 24 h, when the cell culture was 70–80% confluent. One well was transfected with wild type ATP1A3 cDNA, two wells with ouabain resistant, wild type ATP1A3 cDNA and additional 12 wells with the ouabain resistant, mutant ATP1A3 constructs. 24 h post transfection, DMEM medium supplemented with 1 μM ouabain (Sigma) was added to each well. As a control, in one of the two wells transfected with ouabain resistant wild type ATP1A3 cDNA, ouabain was excluded. 48 h after ouabain administration, the cells were
Cells were washed with PBS, harvested with trypsin and centrifuged (210 g, 5 min). Cells were then fixed with 1 ml 4% formaldehyde in PBS at 37 °C for 10 min, and chilled on ice for another 1 min. Permeabilization was done with 90% ice cold methanol for 30 min on ice. Cells were washed and centrifuged twice with incubation medium fixed with 1 ml 4% formaldehyde in PBS and stored at 4 °C overnight. Cells were then incubated with 1% BSA in PBS and stored at 4 °C for 24 h. Following incubation, cells were washed twice with blocking solution, resuspended in PBS and transferred to FACS tubes. Cells were analyzed on a BD LSRII flow cytometer (BD Bioscience) and analysis was performed using FACSDiva software (BD Bioscience) and flowing software 2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland).

2.5. Flow cytometry

Cells were washed with PBS, harvested with trypsin and centrifuged (210 g, 5 min). Cells were then fixed with 1 ml 4% formaldehyde in PBS at 37 °C for 10 min, and chilled on ice for another 1 min. Permeabilization was done with 90% ice cold methanol for 30 min on ice. Cells were washed and centrifuged twice with incubation medium containing 0.5% bovine serum albumin (BSA) in PBS.

Cell pellet was resuspended in 100 μl blocking solution containing 1% BSA in PBS and stored at 4 °C overnight. Cells were then incubated with anti Myc-tag FITC conjugated antibodies (abcam ab1394) diluted 1:100 in blocking solution for 1 h at room temperature. Following incubation, cells were washed twice with blocking solution, resuspended in PBS and transferred to FACS tubes. Cells were analyzed on a BD LSRII flow cytometer (BD Bioscience) and analysis was performed using FACSDiva software (BD Bioscience) and flowing software 2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland).

2.6. Oocyte preparation and microinjection

After linearization of pSGEM plasmids with Nhel, cRNA was synthesized with the T7 mMessage mMachine kit (Ambion, Austin, TX) according to the manufacturer’s protocol. Oocytes were obtained by partial ovariectomy from anesthetized Xenopus laevis. Stage V and VI oocytes were separated after collagenase treatment (0.6–0.8 U/ml collagenase in oocyte Ringer’s solution containing 110 mM NaCl, 3 mM KCl, 2 mM CaCl₂ and 5 mM 4-morpholinosulfonic acid (MOPS), adjusted to pH 7.6) and maintained at 18 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 0.2 mM CaCl₂, 2 mM MgCl₂, 5 mM HEPES, pH 7.5) containing 1 mg/ml gentamicin. A mixture of 50 ng of α and 5 ng of β-subunit cRNAs in a total volume of 50 nl RNAse-free water was injected per oocyte using a Drummond Nanoject II automatic microinjector (Drummond Scientific Company, Broomall, PA). The injection pipette was pulled from borosilicate glass to a tip diameter of approximately 12 μm and backfilled with mineral oil.

2.7. Immunoprecipitation

12 ATP1A3- and ATP1B1-injected and 12 non-injected oocytes were lysed in lysis buffer (20 μl per oocyte) containing 150 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, 5 mM MgCl₂, 5 mM EDTA, pH 7.5 and Complete ultra protease inhibitor cocktail (Roche, catalog No. 05892791001). Cells were resuspended with a pipette tip until they were completely homogenized. For increased lysis, homogenates were incubated on ice for 15 min followed by centrifugation. With the resultant pellet, lysis was repeated. Supernatants have been sonicated for two times 20 s and myc-antibody (Myc-Tag (9B11) Mouse mAb, Cell Signaling, 1:1000) was added and rotary incubated overnight at 4 °C. The next day, 40 μl A/G PLUS-Agarose (Santa Cruz Biotechnology) was added for 3 h. After 3 washing steps with 0.1% Tween 20 in tris-buffered saline (TBST) solution the precipitated proteins were eluted by heating at 95 °C in SDS sample buffer and analyzed by Western blotting as follows. Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane using blotting buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.04% w/v SDS, and 20% v/v methanol. Membrane was blocked in PBST solution with 5% nonfat dried milk and incubated overnight at 4 °C with myc-tag antibody (1:1000). Incubation with an anti-mouse secondary HRP-labelled antibody was followed by detection with Lumi-Light Western blotting substrate (Roche Applied Science).

2.8. Electrophysiology (Two-electrode voltage-clamp)

Two-electrode voltage clamp (TEVC) recordings were performed to measure Na⁺-, K⁺-pump currents 3 days after cRNA injection, using a Turbo TEC-10CD amplifier (NI electronics) at room temperature. The intracellular electrodes had resistances of 0.4–1.2 megohms when filled with 2 M KCl. Prior to recordings, oocytes were incubated in sodium-loading buffer containing 110 mM NaCl, 2.5 mM sodium citrate, and 5 mM MOPS (pH 7.6) for 30–90 min to elevate intracellular Na⁺- concentration (Rakowski et al., 1991). The cells were held at ~60 mV and the stationary Na⁺-/K⁺-pump currents were measured upon solution exchange from K⁺-free buffer containing 90 mM tetra-methylammonium (TMA) chloride, 2 mM CaCl₂, 5 mM BaCl₂, 20 mM tetrathylammonium (TEA) chloride and 5 mM morpholinopropene sulfonic acid (pH 7.6) to a second buffer supplemented with 10 mM KCl (otherwise similar to the first buffer). To determine the currents of the exogenous Na⁺-/K⁺-pumps, pump currents in control (non-injected) oocytes were measured in parallel under the same conditions and the mean values were subtracted from those obtained in cRNA-injected oocytes of the same batch. Data acquisition and analysis were performed with the Pulse-Pulsefit (HEKA Electronics) and IgorPro (WaveMetrics) software packages.
2.9. Structural analysis

The human α3Na+/K+-ATPase is 87% identical the α1 Na+/K+-ATPase from pig, for which crystal structures are available in K+-bound (Morth et al., 2007) and Na+-bound states (Kanai et al., 2013; Nyblom et al., 2013). The mutant sites were mapped on the crystal structure and their functional roles were evaluated on the basis of structural analysis.

2.10. Statistical analysis

Statistical analysis was performed using Graph Pad Prism v 6.01 (GraphPad Software, La Jolla CA). Analysis of multiple groups was done using one-way ANOVA with post-hoc Dunnett’s test. Unless otherwise stated data represent mean ± standard error of the mean (SEM). Asterisks correspond to p-values of < 0.05 (*), > 0.01 (**) or < 0.001 (***)

3. Results

3.1. ATP1A3 expression is affected by some AHC and RDP mutations

We first examined whether RDP and AHC mutations affect the expression level of ATP1A3.

Previous study addressing this question showed that AHC mutations do not affect protein expression whereas some RDP mutations dramatically reduced protein expression, leading to the proposition that AHC mutations modulate pump activity, while RDP mutations exert hypomorphic effects on the pump (Heinzen et al., 2012). However, the results of this study were contradictory with the data of de Carvalho Aguiar et al. (2004). Mutations which reduced protein expression to undetectable levels in Heinzen et al. (2012), demonstrated significant function and expression in de Carvalho Aguiar et al. (2004), and vice versa.

Here we tested protein expression for twelve RDP and AHC mutations expressed in HEK cells, using immunoblot analysis. The transfection efficiency was measured for all constructs using flow cytometry analysis and ranged between 30 and 40% (Table S2). Untransfected HEK cells served as control and showed no detectable band. All constructs showed detectable bands at ~112 kDa, corresponding to ATP1A3 (Fig. 1A). In particular, mutations I274T, T613M and F780L showed high expression, in line with de Carvalho Aguiar et al., (2004). The analysis of band intensities shows significant differences in the expression within the group of AHC mutations (one way ANOVA, p = .0045), with E815K showing the highest expression and D923Y the lowest (Fig. 1B). Differences compared to wild type control were not significant (post hoc Dunnnett test, α = 0.05). The group of RDP mutations showed no significant differences in the expression (p = .95).

These data suggest that some mutations may influence ATP1A3 expression. Different sensitivities of the assays, diverse growing conditions as well as usage of different cell lines may have contributed to the variations between the studies.

We next set out to check how the different mutations affect pump function.

3.2. AHC and RDP mutations reduce cell survival under ouabain challenge

To test for the functional capability of the mutant pumps, we performed a cell survival assay under ouabain challenge. In HEK cells, ouabain inhibits the endogenous α1 Na+/K+-ATPase, leading to cell death within 48 h (de Carvalho Aguiar et al., 2004). Introduction of the two mutations Q108R and N199D was shown to reduce the ouabain sensitivity of Na+/K+-ATPase and to rescue cell survival (Price et al., 1990). Using this approach, we created ouabain resistant ATP1A3 constructs and compared the effect of AHC and RDP mutations on the ability of these constructs to substitute for the ouabain inhibited ATP1A1.

The cell survival rate depends on the concentration of ouabain and on the cell density (Table S3). Untransfected HEK cells, plated at density of 2·10⁴ cells/cm² one day prior to ouabain treatment, demonstrated more than 99% cell death after 48 h in 1 μM ouabain medium, confirming inhibition of ATP1A1. Increasing the cells density to 3·10⁴ cells/cm² reduced the fraction of dead cells to 76%. Because transfection reduces cell viability, cells transfected with wild type ATP1A3 construct (without ouabain resistance mutations), at original density of 3·10⁴ cells/cm², showed more than 96% cell death. Thus, ouabain concentration of 1 μM was chosen for the survival assay.

Cells transfected with ouabain resistant wild type ATP1A3 construct demonstrated survival rate of nearly 30%, matching the transfection efficiency rate. All constructs with AHC or RDP mutations led to elevated cell death compared to wild type control (Fig. 2). The survival rates varied significantly between the mutations (one way ANOVA, p = .021), with the RDP mutation I274T demonstrating the highest survival rate and I758S the lowest, in line with previous report (de Carvalho Aguiar et al., 2004). Our results indicate that both RDP and AHC mutations substantially impair the ATP1A3 function, with no clear difference between the two groups. To directly study how the mutations affect pump current we next turned to electrophysiology.

3.3. AHC and RDP mutations reduce the pump current

Analysis of crystal structures of the α1-isoform suggests that, with the exception of I274N, I274T and I758S, all other mutations studied here should affect ion binding profoundly. Mutations D923N, D232Y, G497R, E815K, F780L are predicted to interfere with the Na⁺ binding site III (Holm et al., 2016). Mutations D801N and D801Y are predicted to interfere with Na⁺ binding (Holm et al., 2016). Backbone carbonyl of V322 coordinates both Na⁺ and K⁺ binding at ion binding site II, and substitution of hydrophobic valine by a polar, charged residue (aspartate) may impair protein conformation and ion binding at site II (Fig. 3B,C). In mutations I274N, I274T and I758S, the change of polarity is predicted to perturb protein folding or activity (de Carvalho Aguiar et al., 2004). A model of the α1-isoform with the location of the mutations, as well as the Na⁺ and K⁺ binding sites, is shown in Fig. 3 and the predicted effect of the mutations on protein structure is summarized in Table 1. Consistent with these predictions, mutations F780L, D923N, α1-T618M (corresponding to T613M) and α1-D804N (corresponding to D801N) were shown to reduce Na⁺ affinity 3- to 200-fold (Einhom et al., 2010; Pedersen et al., 1997; Rodacker et al., 2006; Toustrup-Jensen et al., 2014). However,
physiological data on how ATP1A3 mutations affect pump activity are still limited.

We investigated the effect of the different mutations on α3 Na⁺/K⁺-ATPase activity expressed in *Xenopus laevis* oocytes. The ATP1A3 constructs were injected to the oocytes together with ATP1B1 to ensure membrane localization. Co-expression of ATP1A3 and ATP1B1 was verified using immunoprecipitation (Fig. 4A). Using two-electrode voltage-clamp technique, we measured basal steady state currents in a K⁺ -free bath solution and after the application of extracellular solution containing 10 mM K⁺ (Fig. 4B). The K⁺-stimulated currents could be blocked by the application of 10 mM ouabain to the extracellular solution, which inhibits both the endogenous and the ouabain-resistant exogenous Na⁺/K⁺-ATPases (Fig. 4D). With the exception of mutations I758S, V322D and D801N, a significant reduction in the current was observed for the ATP1A3 mutants compared to wild type ATP1A3 control (one way ANOVA; \( p < .0001 \), Fig. 4C), in agreement with the structural analysis and the anticipated reduction in Na⁺ affinity. The reduction in current was most pronounced for the mutations I274T, I274N and T613M. Interestingly, the currents measured for the I274N mutant were consistently smaller compared to control unmanipulated oocytes, suggesting complete loss of surface expression, lack of activity, or negative effect on the endogenous α1 subunit. We were not able to measure steady state currents, nor reaction after potassium stimulation, in oocytes expressing the AHC specific mutation E815K. The data show no evident difference between the RDP and AHC mutations.

4. Discussion

Mutations in ATP1A3 cause neurological disorders with different phenotypes and various levels of severity. The mechanisms determining disease severity are currently not understood. In this study, we compared the effect of twelve RDP and AHC specific mutations on protein expression, cell survival and pump current, in order to understand how malfunction of the α-subunit is related with the clinical manifestation. Because all known ATP1A3 mutations are heterozygous, we used HEK cells and oocytes as expression systems where only the mutant allele is expressed, to facilitate the comparison. While AHC phenotype is often more severe compared to RDP, we have not found a clear distinction between the two mutation groups neither in the expression nor in the functional studies, although AHC mutations cluster more to the transmembrane domain (Heinzen et al., 2014). Our data suggest that the level of functional impairment of the α3 subunit by itself, does not
| Mutation | Phenotype | Localization | Predicted effect on structure and function | Change in Na$^+$ affinity |
|----------|-----------|--------------|------------------------------------------|--------------------------|
| I274T RDP M3, stalk | I274 is part of a hydrophobic interaction network near the interface of the M3-M5 helices and the P domain (including I279). | High (+86%) | Low (< 60%) | Reduction in pump current (rat 30%, pig 10%) |
| I279A | Similar to I274, the charge of polarity could disturb protein folding or activity. | High (+67%) | Intermediate (−30%) | Reduction in pump current (rat 30%, pig 10%) |
| I758S RDP M5, stalk | Similar to I274, the charge of polarity could disturb protein folding or activity. | High (+89%) | Low (< 30%) | Reduction in pump current (rat 30%, pig 10%) |
| F780L RDP M5, membrane domain | F780 forms stacking interactions with aromatic residues lining the extracellular end of M3, M4 and M5 in the E1 state. F780 is placed just above residues contributing to site I (N873 and E876) and site III (Y868, T871 and S872). | High (+89%) | Low (< 30%) | Reduction in pump current (rat 30%, pig 10%) |
| D801N AHC M6, ion binding site I and II | D801 forms stacking interactions with aromatic residues lining the extracellular end of M3, M4 and M5 in the E1 state. D801 is placed just above residues contributing to site I (N873 and E876) and site III (Y868, T871 and S872). | High (+86%) | Intermediate (−30%) | Reduction in pump current (rat 30%, pig 10%) |
| D804N, D807 | D801Y AHC/RDP Intermediate (+72%) | Intermediate (−30%) | Reduction in pump current (rat 30%, pig 10%) |
| E815K AHC L6/7 | E815K participates in an extensive hydrogen bond/ionic interaction network involving L6/7, L8/9, M5 and the C-terminus. This network controls the movement of M5 to ensure normal Na$^+$ binding and selectivity. Change of charge is expected to distort the interaction network and negatively impact the Na$^+$ binding (4). | High (+86%) | Intermediate (−30%) | Reduction in pump current (rat 30%, pig 10%) |
| D923Y AHC High (+81%) | Low (< 30%) | Reduction in pump current (rat 30%, pig 10%) |
| D923N AHC/RDP M8, ion binding site III Neutralization of charge is expected to interfere with Na$^+$ coordination, and the bulkiness of tyrosine will conceivably disturb binding site III. K$^+$ binding is likely intact (4). | High (+86%) | Intermediate (−30%) | Reduction in pump current (rat 30%, pig 10%) |
| G947R AHC M9, membrane domain | The bulky side chain and charge of the arginine mutation (pointing towards Y768) will probably perturb ion binding site III, affecting Na$^+$ interaction (4). | High (+86%) | Intermediate (−30%) | Reduction in pump current (rat 30%, pig 10%) |
| Table 1

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exclusively determine the disease severity.

All the mutant constructs were successfully expressed in HEK cells, with some variations in the expression levels as reflected by immunoblot analysis. Immunostaining confirmed ATP1A3 expression and additionally revealed localization to intracellular compartments (Fig. S3). Our data show significant differences in the expression within the group of AHC mutations studied but not between the RDP mutations (Fig. 1). A previous study in COS-7 cells, examining different set of mutations, has reported opposite results (Heinzen et al., 2012). Together this suggests that some mutations might influence protein expression for both phenotypes.

All the mutations studied demonstrated poor survival after incubation with ouabain compared to wild type control, with no difference between RDP and AHC mutations. Because all ATP1A3 constructs demonstrated high expression level, we conclude that the reduced survival is a result of functional impairment.

When expressed in oocytes, 8 of the 12 mutations led to a significant reduction in the pump current. A previous study demonstrated lower pump current for three AHC mutations (D801N, G947R, E815K) expressed in oocytes, but despite the clinical heterogeneity, the level of reduction was similar (Li et al., 2015). Here we expanded the pool of mutations to include RDP and AHC mutations. Our data show large differences in the pump current between the mutations. Three of the mutations demonstrated pump current similar to control, while associated with classical AHC or RDP phenotype. Complete reduction of pump current was measured for mutation I274N, which has classical AHC phenotype with no apparent particularities (Rosewich et al., 2012). The reduction in pump current showed no correlation with disease severity. This conclusion has two caveats: 1) Li et al., 2015 have shown that the AHC mutations E815K, D801N and G947R have a dominant negative effect when expressed in oocytes together with a normal copy of ATP1A3. Because we did not inject the oocytes with an additional wild type construct, the reported variations in the pump current might change in the heterozygous state if dominant negative effect is differentially exerted. 2) Because we could not confirm that the mutant constructs are expressed on the oocyte membrane at similar densities, differences in the pump currents might be associated not only with impairment of pump activity but also with altered expression. Although the mutations E815K and D923N do not show evidence for misfolding and aberrant targeting to the membrane (Arystarkhova et al., 2019), additional experiments are needed to investigate other AHC and RDP mutations.

Studies in mice have shown that different mutations in ATP1A3 can disrupt, to variable extent, protein expression. The AHC mutation I810N showed no difference in immunoreactivity in whole brain homogenates (Clapcote et al., 2009) whereas mice heterozygous for a point mutation in ATP1A3 intron 4 (Atplagm11azeq) show strong (~60%) reduction of α3 expression in the hippocampus (Moseley et al., 2007). In mice heterozygous to the RDP/AHC D801Y, the expression of α3 in the cerebellum was found to be 20% lower compared to wild type (Isaksen et al., 2017), in agreement with our expression data for this mutation. Notably, while not affecting expression level, mutation I810N

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Fig. 4. RDP and AHC mutations reduce the forward cycling current in oocytes. (A) Immunoprecipitation analysis of oocytes injected with myc-tagged ouabain resistant wild type ATP1A3 and ATP1B1 cRNA. Unmanipulated oocytes were used as control (left lane). The blot shows bands at ~110 kDa and ~ 40 kDa, corresponding to precipitated ATP1A3 and ATP1B1, respectively. An additional 55 kDa band represents the heavy chain of the antibody used for the precipitation. (B) Representative current traces measured in oocytes in response to the application of 10 mM K+ to the extracellular solution. (C) Average steady state currents measured in oocytes for the different mutant constructs, demonstrating significant differences compared to the wild type construct (mean ± SEM, *p > .05, **p > .001, ***p > .0001). (D) Representative current traces demonstrating that the K+ stimulated currents were blocked by the application of 10 mM ouabain.
was reported to reduce brain total ATPase activity by ~40%, suggesting inactivation of the mutant α3 subunit (Clapcote et al., 2009). In At-
pA1α2m1Ling mice, on the other hand, which showed marked reduction in expression level, only ~15% reduction of total brain ATPase activity was measured (Kirshenbaum et al., 2011). While this suggests that high expression level might be associated with lower enzyme activity, further evidence is needed to confirm this apparent relationship.

Surprisingly, mutation I758S that led to the highest death rate under ouabain challenge, showed no effect on the pump current, whereas mutation I274T led to the lowest death rate but dramatically reduced the pump current. This suggests that the mechanisms involved in the regulation of apoptosis by impairment of the Na+/K+ -ATPase activity might not be directly linked to the change in the Na+ and K+ gradients. How exactly ouabain triggers cell death is not entirely understood. Treatment with ouabain was shown to increase intracellular Na+ and Ca2+ concentrations, blunting the accumulation of mitochondrial Ca2+ thus leading to mitochondrial dysfunction, decrease in the cellular ATP content (Alonso et al., 2013; Takase et al., 2017) and apoptosis. Other cytotoxic mechanisms of ouabain include the formation of reactive oxygen species (ROS) which damages DNA (Liu et al., 2000), and autophagy (Meng et al., 2016; Trenti et al., 2014). Interestingly, in renal epithelial cells, cell death was induced by ouabain independent of changes in the Na+ and K+ fluxes, leading to the hypothesis that the interaction with ouabain induces conformational change which triggers a death signal (Oriol and Hamet, 2006; Pchetski et al., 2003).

In oocytes expressing the EB1SK mutation, one of the most common AHC mutations (Heinzen et al., 2012; Rosewich et al., 2012) which has been associated with the most severe AHC phenotype (Sasaki et al., 2014; Violeet al., 2015), we could not measure steady state or K+-stimulated currents. One hypothesis explaining this result is that this mutation changes the structure of ATP1A3 in a way that communica-
tion between the cytoplasmic and extracellular gates of the Na+/K+-pump is affected. Normally, Na+/K+ -ATPases, as any active transporter, acts with strict coupling between the two gates to ensure that each gate opening is interspersed by an occluded state (Gadsby, 2009) disfavoring rapid backflow. It has been shown that a disruption of the tight coupling upon palytoxin binding, can allow both gates to be occasionally open at the same time, thus transforming a Na+/K+-pump into a de-
polarizing channel. These Na+/K+ -ATPase-pump-channels could still react to extracellular K+ and cytoplasmatic ATP but the size of the current through a single palytoxin-bound pump-channel (more than a million Na+ ions per second flow through each pump) was much higher than the one measured in intact Na+/K+ -ATPases (Artigas and Gadsby, 2003; Gadsby, 2009). If AHC mutants like EB1SK can allow simultaneous opening of the intracellular and extracellular gates, similar to palytoxin, it will transform the pump into a channel, and the cell membrane might become too leaky to enable a measurement of steady state current. While such channel gain of function would likely be asso-
ciated with a depolarized resting membrane potential, as measured in AHC neurons (Simmons et al., 2018), further investigation is needed to support this hypothesis.

Which factors can account for the phenotypic diversity of ATP1A3 mutations? In large familial cases of RDP, the phenotype has been re-
ported to range from non-manifesting to severe for the same mutation (Dobyns et al., 1993; Pittock et al., 2000). In familial cases of AHC, mutations? In large familial cases of RDP, the phenotype has been re-
ported to range from non-manifesting to severe for the same mutation (Simmons et al., 2018), further investigation is needed to determine if there is an underlying, common mechanism for AHC mutant forms that distinguish them from RDP mutants. Finding such char-
acteristics may also guide the identification of a cure.

**Funding**

We would like to acknowledge Corinna Dickel for expert technical assistance.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2020.105012.

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