The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/80335

Please be advised that this information was generated on 2017-07-27 and may be subject to change.
Mutations in three different genes have been implicated in familial hemiplegic migraine (FHM), two of them code for neuronal voltage-gated cation channels, CACNA1A and SCN1A, while the third encodes ATP1A2, the \( \alpha_2 \)-isoform of the Na\(^+\)/K\(^+\)-ATPase's catalytic subunit, thus classifying FHM as an ion channel/ion transporter disorder. The Na\(^+\)/K\(^+\)-ATPase maintains the physiological gradients for Na\(^+\) and K\(^+\) ions and is therefore critical for the activity of ion channels and transporters involved in neurotransmitter uptake or Ca\(^{2+}\) signaling. Diverse functional abnormalities have been identified for disease-linked ATP1A2 mutations, which reach far beyond simple loss-of-function. We have shown recently that ATP1A2 mutations frequently lead to changes in the enzyme's voltage-dependent properties, kinetics or apparent cation affinities. Here, we present functional data on a so far uncharacterized set of ATP1A2 mutations (G301R, R908Q and P979L) upon expression in *Xenopus* oocytes and HEK293FT cells, and provide evidence for a novel pathophysiological mechanism. Whereas the G301R mutant was inactive, no functional changes were observed for mutants R908Q and P979L in the oocyte expression system. However, the R908Q mutant was less effectively expressed in the plasma membrane of oocytes, making it the first missense mutation to result in defective plasma membrane targeting. Notably, the P979L mutant exhibited the same cellular expression profile as the wild-type protein, both in *Xenopus* oocytes and in transfected HEK293FT cells grown at 28°C, but much less P979L protein was found upon cell growth at 37°C, showing for the first time that temperature-sensitive effects on protein stability can underlie ATP1A2 loss-of-function.

**Introduction**

Familial hemiplegic migraine (FHM) is a rare and severe autosomal-dominant form of migraine with aura, which is characterized by some degree of reversible motor weakness during the aura phase. FHM type 2 (FHM2) is caused by mutations in ATP1A2, the gene coding for the human Na\(^+\)/K\(^+\)-ATPase \( \alpha_2 \)-subunit. Mutations in two other genes have also been implicated in the disease, namely in CACNA1A (FHM1) and in SCN1A (FHM3), which code for neuronal voltage-gated Ca\(^{2+}\) or Na\(^+\) channels, respectively, suggesting that perturbations in neuronal excitability underlie the neurological phenotype. Besides the involvement in FHM, ATP1A2 mutations have been described in sporadic forms of hemiplegic migraine (SHM) and also in more general types of migraine with or without aura (MA/MO) or even in basilar migraine (BM). Moreover, there is significant clinical overlap to other neuropathologies involving mental retardation, seizures or forms of epilepsy like benign familial infantile convulsions (BFIC) (refer to the paper by Morth and colleagues for a complete list). It is assumed in current disease models that most of the aura symptoms are directly linked to the phenomenon of cortical spreading.
Defective targeting or protein stability of ATP1A2

Results and Discussion

Upon expression of WT and mutant human Na⁺/K⁺-ATPase α₂-subunits together with the β₁-subunit in Xenopus oocytes, we measured stationary Na⁺/K⁺ pump currents and ouabain-sensitive uptake of Rb⁺. Figure 1A shows that the stationary pump currents of the P979L mutant were slightly smaller compared to WT, whereas a reduction by about 80% was found for the R908Q mutant, and the currents measured on oocytes expressing the G301R mutant were indistinguishable from baseline fluctuations. Rb⁺ uptake was nearly identical for ATP1A2 WT and P979L, and largely reduced for mutants R908Q and G301R. To explore more subtle functional consequences, we measured the voltage and [K⁺]ₜₐₜ dependence of K⁺-stimulated pump currents, and determined kinetics and voltage dependence of change movement from ouabain-sensitive transient currents mediated by Na⁺/K⁺-ATPase under extracellularly high [Na⁺] and K⁺-free conditions. Although stationary currents of the R908Q mutant were small, a sufficient number of cells could be analyzed for this construct. Compared to the WT properties determined previously (see Fig. 3A in Tavraz et al.¹⁰), the stationary I-V curves of the R908Q mutant (Fig. 1C) were very similar. The same was true for the P979L mutant (Fig. 1D), and also the voltage-dependent Kₜₐₜ values for the [K⁺]ₜₐₜ dependence of pump currents were very similar to WT for R908Q (Fig. 1E) and P979L (Fig. 1F). Furthermore, the parameters V₀.₅ and zₚ from fits of a Boltzmann function to the Q-V distributions of translocated charge and turnover numbers were not different from the WT values for mutants R908Q and P979L (Table 1). For the R908Q mutant, the reciprocal time constants from transient currents were also identical to WT (Fig. 1G), whereas slightly elevated rate constants were found for P979L (Fig. 1H). In summary, ion transport activity, as assessed by pump current and Rb⁺ uptake measurements, was almost completely lost for mutant G301R, largely reduced for R908Q (albeit without detectable functional changes on a molecular level), but unaltered for mutant P979L.

At this point, it was critical to determine protein expression levels and to monitor the relative amounts of ATP1A2 protein in the plasma membrane. Figure 2A shows that for the P979L mutant the amounts of α₂-subunit protein in total membrane as well as plasma membrane fractions were similar to WT. However, protein abundance in the plasma membrane fraction was slightly smaller for the G301R mutant, and for R908Q severely reduced, although the amounts of α₂-subunit protein in the total cellular membrane fractions were identical to WT (Fig. 2B).

Therefore, loss of activity for the G301R mutant can be attributed to a normally targeted but inactive enzyme, as found previously for mutations R764P and W887R. Gly301 resides in the central portion of transmembrane helix M3 (Fig. 3) where it closely approaches helix M4 around Leu322 and Ile325. Thus, mutation to a bulky arginine is likely to distort the local structure, and since the adjacent residue Glu332 is part of the cation binding pocket, this putative disruption may interfere with cation coordination or transport. For the R908Q mutation, deficient plasma membrane targeting can account for loss-of-function, although the mutant enzyme itself is fully functional.
Defective targeting or protein stability of ATP1A2

Membrane expression has previously been observed for the S966fs frame shift mutation and the deletion/insertion mutation del(KO35-S940)ins(I),\textsuperscript{10} which represent severe disruptions of the primary structure and likely cause protein misfolding and degradation. Notably, R908Q is the first FHM2 missense mutation to result in defective plasma membrane targeting. Arg\textsuperscript{908} resides in the loop between transmembrane segments M8 and M9 and may be critical for structural integrity of the extracellular face of the Na+/K+-ATPase α-subunit, since the terminal amino group of this arginine is able to form an H-bond (~3.5 Å) to the backbone carbonyl oxygen of Leu\textsuperscript{797} in the M5-M6 loop (Fig. 3). Reduced plasma membrane trafficking can also explain the previously published results from ouabain survival assays, in which a severe impairment of cell viability was found for cells expressing the R908Q mutant, although the amount of total cellular α\textsubscript{2}-subunit protein was not changed compared to WT.\textsuperscript{12}

Since the P979L mutant did not show any functional alterations in our oocyte experiments at room temperature (20–22°C), we asked, whether a difference in the temperature dependence of the ion-pumping rate could lead to divergent activity at body temperature. This could happen either if the activation energy of the rate-limiting step for ion pumping by the P979L mutant protein would be different from that of the WT protein, or if at elevated temperature, another partial reaction of the catalytic cycle with a different temperature dependence would become rate limiting. To explore this possibility, we measured the temperature dependence of stationary pump currents between 18°C and 34°C on oocytes expressing ATP1A2 WT or the P979L mutant. Analysis of the resulting Arrhenius plots (data not shown) yielded very similar activation energies for WT and P979L. Two temperature regimes were generally observed with a transition temperature around 26°C (see Table 1). Such 'breaks' in Arrhenius plots have previously been observed for e.g., native Na+/K+-ATPase from rat cardiac myocytes\textsuperscript{16} and have tentatively been assigned to a phase transition within the lipidic cell membrane. However, also these measurements exclude the possibility that

![Figure 1. Functional properties of ATP1A2 mutants.](image-url)
Defective targeting or protein stability of ATP1A2

### Table 1: Electrophysiological properties of ATP1A2 mutants

<table>
<thead>
<tr>
<th></th>
<th>$V_{0.5}$ (mV)</th>
<th>$z_q$</th>
<th>Turnover (s$^{-1}$)</th>
<th>N</th>
<th>$E_A$ (kJ/mol) 18°C–26°C</th>
<th>$E_A$ (kJ/mol) 26°C–34°C</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.3 ± 3.5</td>
<td>0.82 ± 0.03</td>
<td>12.8 ± 3.0</td>
<td>18</td>
<td>132 ± 14</td>
<td>87 ± 15</td>
<td>5</td>
</tr>
<tr>
<td>G301R</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>R908Q</td>
<td>8.3 ± 3.5</td>
<td>0.82 ± 0.03</td>
<td>13.0 ± 4.7</td>
<td>8</td>
<td>N/D</td>
<td>N/D</td>
<td>5</td>
</tr>
<tr>
<td>P979L</td>
<td>3.8 ± 7.1</td>
<td>0.79 ± 0.04</td>
<td>18.6 ± 5.4</td>
<td>21</td>
<td>124 ± 6</td>
<td>80 ± 2</td>
<td>5</td>
</tr>
</tbody>
</table>

Parameters from fits of a Boltzmann function to the voltage-dependent distributions of charge from transient currents recorded in voltage jump experiments under extracellularly high (Na$^+$/K$^+$)-free conditions. $V_{0.5}$ values denote midpoint potentials, $z_q$ the slope factors (equivalent charges), and $E_A$ activation energies from Arrhenius plots derived from measurements of the temperature dependence of pump currents. N is the number of cells included in analysis. N/A, not applicable; N/D, not determined.

**Figure 2.** Expression of ATP1A2 constructs in Xenopus oocytes and HEK 293FT cells. (A and B) Representative western blots of samples from plasma membrane (PM) and total membrane (TM) fractions from oocytes expressing various ATP1A2 constructs. Samples equivalent to one oocyte were loaded per lane. Uninjected oocytes served as control. (C) Expression of ATP1A2 WT and mutants upon transient transfection in HEK 293FT cells and growth at permissive (28°C) and non-permissive (37°C) temperature. Expression of the Na$^+$/K$^+$-ATPase $\alpha_2$-subunit and $\beta$-actin (used as loading standard) was assessed by western blotting of whole cell lysate samples, each containing 15 µg of total protein. As an additional control for the consistency of the preparation, the non-disease-related polymorphism I883L$^{13}$ was also investigated using this assay. This mutant was expressed in a similar manner as the WT enzyme. Experiments were repeated three (A and B) or five (C) times, with identical results.

**Figure 3.** Location of mutated residues within the Na$^+$/K$^+$-ATPase structure. (A and B) Structural representation of the Na$^+$/K$^+$-ATPase according to protein data bank (PDB) structure entry 3B8E with amino acids numbered according to human ATP1A2. The numbering according to the rat Na$^+$/K$^+$-ATPase $\alpha_1$-subunit, which underlies the 3B8E structure, is given in brackets below. Residues mutated in this study are shown in ball and stick representation, two red spheres indicate Rb$^+$ ions within the cation-binding pocket as identified in the crystal structure. Structural elements outlined in color: Helix M3 (olive) contains Gly$^{301}$ (Gly$^{296}$), helix M8 (light orange) contains Arg$^{908}$ (Arg$^{904}$), the loop between helix M9 and M10 (magenta) contains Pro$^{979}$ (Pro$^{975}$). The central helix M5 is shown in green. It contains Leu$^{799}$ (Leu$^{793}$), whose backbone carboxyl group is the only candidate for the formation of an H-bond (red dashed line) to Arg$^{908}$ within a radius of 5 Å. (A) Side view of the transmembrane part of the Na$^+$/K$^+$-ATPase $\alpha_2$-subunit. (B) View from the extracellular side roughly along helix M5. In this panel, the $\beta$-subunit is also depicted (violet).
Defective targeting or protein stability of ATP1A2

A change in the temperature dependence of the ion pumping rate underlies loss-of-function of the P979L mutant.

Since no changes in molecular function for the P979L mutant could be observed in oocytes we explored other expression systems to reveal abnormalities of the mutant protein. Preliminary experiments using HeLa cells to test whether the P979L mutant was able to confer cell survival in ouabain-containing media showed reduced cell viability compared to WT-transfected cells, yet this effect was subject to substantial batch variations (data not shown). However, we took this as first indication that expression in mammalian cells and/or cell growth at 37°C could aid to discriminate between ATP1A2 WT and the P979L mutant. Pro979 resides within the short extracellular M9-M10 loop, where it ensures a rather rigid local structure, which is likely to be relaxed upon substitution for a leucine. This could potentially lead to a less effective folding or even misfolding of the structurally important carboxy-terminal region. It has been shown that transmembrane helices M5-M10 are problematic for membrane integration of the Na+/K+-ATPase α-subunit upon biosynthesis at the ER. These six helices probably have to be inserted into the membrane as a single block and may present several putative degradation signals when erroneously exposed to the cytoplasm. However, we did not find any indication for misfolded P979L protein in Xenopus oocytes, but it is known that folding defects of mutant proteins can be revealed in a temperature-dependent fashion. The most thoroughly investigated example is probably the ΔF508 mutation in the CFTR chloride channel, the most frequent sequence variant in cystic fibrosis. Cellular quality control systems for correctly folded membrane proteins are stringently applied to nascent CFTR proteins. It has been shown that the ΔF508 mutant is efficiently and rapidly degraded before exiting the ER in mammalian cell cultures. This effect was shown to be temperature-sensitive, which easily explains why CFTR-ΔF508 could be functionally expressed in Xenopus oocytes. We therefore investigated, whether temperature-dependent protein degradation could occur for the P979L mutant. For this purpose HEK293FT cells were transfected with cDNA of the WT or mutant Na+/K+-ATPase α2-subunits plus β1-subunits. Cell batches were split 24 hours after transfection and subsequently grown at 28°C and 37°C. Figure 2C shows a western blot of cell lysates from such cell cultures. These experiments consistently (N = 5) showed that the amount of P979L mutant α2-subunit was identical to WT at 28°C, whereas a largely reduced protein abundance was found at 37°C. These results for the first time provide evidence, that a temperature-dependent effect on protein stability might underlie the loss of Na+/K+-ATPase function in FHM2 and expands the variety of abnormalities caused by mutations identified in this disease. It will be challenging to reveal the cellular mechanisms and pathways underlying the temperature-dependent degradation of the Na+/K+-ATPase α2-subunit.

The spectrum of molecular phenotypes of ATP1A2 mutations may still be incomplete. For instance, it has not been tested whether FHM2 mutations might disrupt interactions with e.g., adapter proteins. In this context, it is notable that the LQT4 subtype of the cardiac long QT syndrome is caused by mutations in ankyrin B, which is required for recruitment of certain ion transporters (including the Na+/K+-ATPase α2-subunit) to specific microdomains of cardiomyocyte membranes to coordinate spatio-temporal Ca2+ signalling. Thus, it will be interesting to see whether, in addition to the already established complexity of molecular phenotypes, changes in subcellular localization can underlie ATP1A2 loss-of-function.

Concluding Remarks

Our results show that assays to investigate the consequences of FHM2 or SHM mutations in heterologous expression systems, which do not include determination of plasma membrane protein, are insufficient to characterize the origin of a possible loss-of-function at the level of the protein. To discriminate between molecular dysfunction and structure-folding-related phenomena, it would be helpful to combine ouabain survival assays, which are commonly utilized as a first test for functional defects in studies describing novel ATP1A2 mutations, with biotinylation studies that also quantify the amount of plasma membrane protein. The mutations R908Q and P979L are the first examples within the ATP1A2 protein that result in completely functional enzymes, which are most likely defective in targeting to the plasma membrane (R908Q) or subject to premature degradation at body temperature (P979L). These observations expand the spectrum of molecular phenotypes of ATP1A2 mutations in sporadic and familial hemiplegic migraine and call for the inclusion of cell biological assays to clarify the correlation between the cellular fate of the protein and primary disease-causing processes.

Materials and Methods

Xenopus oocytes were used for expression of the ouabain-insensitive (IC50 in mM range achieved by mutations Q116R and N127D) human Na+/K+-ATPase α2-subunit (referred to as WT ATP1A2) and mutants thereof, together with human β1-subunits. Measurements of stationary pump currents, pre-steady state currents, Rb+ uptake, and plasma membrane or total cellular membrane preparations were carried out as described.

Expression in HEK-293FT cells and temperature shift assays. Amino-terminally HA-tagged human Na+/K+-ATPase α2-subunits were expressed together with human β1-subunits in HEK-293FT cells upon transient transfection of 1:1 cDNA mixtures using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Cells were grown to about 70% confluence in 10 cm Petri dishes in DMEM/Glutamax medium + 10% fetal bovine serum and 10 μM ouabain. Water-transfected cells served as control. Twenty-four hours after transfection, the cell charges were split and incubated separately at 28°C or 37°C, respectively, for an additional 24 hours. Subsequently, cells were harvested and subjected to whole cell lysis. After determination of total protein concentration in these total lysates using a bicinconinic acid assay (Perbio, Bonn, Germany), samples containing 15 μg of total protein were separated on sodium-dodecylsulfate polyacrylamide gels by electrophoresis and subsequently transferred to nitrocellulose membranes. The HA-tagged α2-subunits were detected with primary rat monoclonal anti-HA antibody 3F10 (Roche Molecular Biochemicals, Mannheim, Germany). β-actin was detected as a loading standard.
using mouse monoclonal anti-β-actin antibody AC-15 (Sigma, Deisenhofen, Germany). Secondary antibodies were horseradish peroxidase conjugates against rat or mouse (Promega, Mannheim, Germany), and the ECL chemiluminescence reagent (Pierce) was used for imaging on a Kodak Biomax film (Sigma, Deisenhofen, Germany).

Acknowledgements

The authors thank Janna Lustig for excellent technical assistance in cell culture experiments. Work was supported by the Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. and grants of the Deutsche Forschungsgemeinschaft (SFB 740, SFB 429, and Cluster of Excellence “Unifying Concepts in Catalysis”).

References