Format for ANSWERING REVIEWERS

Berlin, 9 April 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 7729-review.docx).

Title: Functional analysis of human Na^+/K^+-ATPase familial or sporadic hemiplegic migraine mutations expressed in *Xenopus* oocytes

Author: Susan Spiller, Thomas Friedrich

Name of Journal: *World Journal of Biological Chemistry*

ESPS Manuscript NO: 7729

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated.

2 The manuscript has been proof-read by an English native speaker.

3 Revision has been made according to the suggestions of the reviewers

(1) Reviewer #1 (01004042): Comment to authors

The aim of this work is Functional characterization of ATP1A2 mutations that are related to Familiar Hemiplegic Migraine (FHM). The approach is to express mutants of this protein in oocytes and examine the location and function of this protein. In general the work is straight forward and interesting. There are minor errors in writing. However, there is a major flaw in this work. "To reduce ouabain sensitivity, the mutations Q116R and N127D were introduced in the α2-subunit. This construct is herein referred to as WT." Thus WT is already mutated. In the judgment of this reviewer, one cannot conclude that the consequences of the mutations would have been the same if the control was not already mutated even though the initial mutations are in a different domain of the protein. This raises doubt on validity of any of the conclusions of this work.

Response:

Since animal expression cell lines such as *Xenopus laevis* oocytes, as well as HEK, CHO or HeLa cells, all express an endogenous Na^+/K^+-ATPase, measures have to be taken to discriminate the activity of the heterologously expressed Na^+/K^+-ATPase α_2-subunit from the endogenous pump. Since the endogenous Na^+ pump forms (apart from that in rats) are highly ouabain-sensitive (with an IC_{50} in the low micromolar range), the overexpressed human α_2-pump is conferred a reduced ouabain sensitivity by the mutations Q116R and N127D (see publication by Price and Lingrel, cited as ref. 28). This strategy is used by all researchers who have studied the effects of mutations in Na^+/K^+-ATPase by electrophysiological techniques upon heterologous expression so far (see ref. 12, 17, 40 and 42). It is well established and accepted by the scientific community that these two mutations do not interfere with catalytic and cation transport properties of the Na^+/K^+-ATPase. In a publication by Vedovato and Gadsby (ref. 18), functional consequences of different mutations that have an impact on ouabain sensitivity were analyzed, but the observed differences relate to a peculiar H^+ transport mode that only occurs under unphysiological conditions (absence of

extracellular Na^+ and K^+).
The so-mutated "WT enzyme" (mutations Q116R and N127D) is sometimes referred to as "RD enzyme" to indicate the mutations in the ouabain binding site. It must be emphasized that the RD mutations provide an appropriate background to study the effects of further mutations of the Na^+/K^+-ATPase. It is otherwise impossible to unambiguously identify the activity of the overexpressed against the endogenous enzyme. We admit that using the term "WT" without mentioning the Q116R/N127D mutations might be misleading to scientists who are not familiar with this issues. Therefore, we have clearly indicated the RD mutations in the materials and methods section and changed the expression "WT" to "RD-WT" in text, figure labels and legends during revision.

(2) Reviewer #2 (00197104): Comment to authors

In continuation to previous work, the authors have studied the functional impact of 7 new mutations in the alpha2 subunit of the NaK ATPase. They show that these mutations that are related to familial and sporadic hemiplegic migraine have different effects on enzyme activity and trafficking to the plasma membrane. The technical quality of the work is good, and the data are convincing and well discussed. Minor comments: - A careful rereading is necessary to correct a few typos (for example familial instead of familiar in familiar hemiplegic migraine, aim and introduction sections). - In the paragraph "Functional consequences" of the discussion ref 26 has been used instead of ref 15 (about P979L mutation).

Response:
We thank the reviewer for indicating this type of misspelling. We have corrected "familiar" to "familial" throughout the manuscript and corrected the reference to the paper reporting the effects of the P979L mutation.

(3) **Reviewer #3 (00503000): Comment to authors**

The authors study the functional consequences of seven mutations related to familial and sporadic hemiplegic migraine, in the alpha2 subunit of the NA, K ATPase. This is an interesting work that complements previous works of the authors. I consider that some issues needs to be resolved before publication.
Major comments: -Due to the methodological strategy employed, the authors are studying mutations in an already mutated protein. To strengthen their conclusions, the author need to provide some evidence that the mutations introduced in the WT have no further consequences in the protein and the mutants they are studying -Western blot analyses lack a loading control - The authors must provide statistical analyses of their data.
Minor comments: -In the title, the abbreviation should be changed for the complete name of the disease -In the whole manuscript, familiar must be changed for familial.

Response:
Concerning the mutations introduced in the WT enzyme to reduce ouabain sensitivity: Please see our response to the comments of Reviewer #1.
Regarding the loading control in western blots: Since the analysis of protein expression is based upon a well-defined number of cells (*Xenopus* oocytes are large and homogenous in size, typically 8 to 12 cells are used for one plasma membrane expression sample), loading the equivalent of a certain number of cells into a western blot lane does not require an internal expression standard such as beta-actin. This has been shown in our previous work (Tavraz et al. cited as ref. 15, see Figure 2C therein). Thus, loading the equivalent of a certain number of oocytes on an SDS gel already provides an internal loading standard. We have emphasized this issue in the materials and methods section as well as in the legend to Fig. 6.
Statistical analysis: We have carried out statistical analysis based on the Student's *t*-test for independent samples. The significance level $P>0.05$ is now indicated as an asterisk above the data points reaching this significance level. The materials and methods section, figures and figure legends have been updated,

accordingly.
We have changed the title to replace "FHM" by familial hemiplegic migraine" and exchanged "familiar" for "familial" throughout the manuscript.

(4) **Reviewer #4 (00504439): Comment to authors**

The authors provide a firm and well performed studies on WT and mutant pumps, I have very few comments, since the authors did not number the MS I will try to inform about the location of my points. 1- Introduction page, the authors mention that K binding stimulates dephosphorylation and conformational change E2 to E1 without mentioninig the role of cytoplasmic ATP in inducing the E1 form. 2-Results, under electrogenic Na,Na exchange. and not "und" 3-From the abstract I have got the impression that K1003E has no impact on pump function, yet the mutation produces a clear shift in Na,Na exchange, because the affinity of the third Na site can be changed independently, it would be more precise to say that K1003 had no impact on K interaction.

Response:
Point 1: During revision, we introduced a remark concerning the ATP dependence of the E2→E1 transition in the introduction section.
Point 2: This typo has been corrected.
Point 3: We thank the reviewer for this helpful suggestion. We have changed the sentence in the abstract to "the K1003E mutation had no impact on K^+ interaction", accordingly.

3 References and typesetting were corrected.

Thank you again for publishing our manuscript in the *World Journal of Biological Chemistry*.

Sincerely yours,

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