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A variant of KCC2 from patients with febrile seizures impairs neuronal Cl- extrusion and dendritic spine formation

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Transfer Note:

Please note that this manuscript was originally submitted to the EMBO Journal where it was peer-reviewed and revised. It was then transferred to EMBO reports with the referees' comments attached, as well as the authors' responses. (Please see below)

Editor: Esther Schnapp

Transfer of revised paper with referees' commtens from the EMBO Journal - authors' response 09 March 2014

Referee #1:

In their revised manuscript, Puskarjov and colleagues have addressed some of my concerns regarding the phenotypic characterization of a missense KCC2 variant identified in patients with febrile seizures. They now provide evidence that the R952H variant leads to reduced KCC2 surface expression in heterologous cells. This new data somehow link previous data on chloride homeostasis and spine maturation. They also made a number of marginal changes to the text and title to suggest this KCC2 variant may represent a susceptibility (but not causal) factor to febrile seizures. In their rebuttal, the authors also make a careful and witty selection of the reviewers' concerns to emphasize our appreciation of the potential impact of their work. Although I do think

this report will have a significant impact in the field of chloride transport/epilepsy, as stated in my original review, I still wonder whether the EMBO Journal (which usually publishes more thorough, mechanistic studies of cellular processes of general interest) is most appropriate for publication of this kind of study.

Specific points

1. As suggested in my original review, work from others have shown KCC2 overexpression in immature neurons (before KCC2 expression normally occurs) leads to several alterations that may complicate the interpretation of the present data, including reduced GABA signaling. The authors state that they 'have presently and previously (Fiumelli et al., 2013) demonstrated that the effect of transfecting cortical neurons with KCC2 in vivo is an increase in the number glutamatergic synapses. In the study by Chudotvorova et al., KCC2 transfection of cultured neurons was reported to increase GABAergic but not glutamatergic synaptogenesis, pointing to a fundamental difference between the effects of KCC2 overexpression seen in their studies and those observed in vivo'. This is a somewhat biased statement, as the Fiumelli paper simply did not test GABAergic function. Hence the authors simply cannot conclude on this point. In addition, the presence of TTX will prevent action potential generation, as stated by the authors, but not GABAA receptor tonic activation which may significantly affect input resistance (Brickley et al J Physiol 1996). In this context, it is remarkable that dendritic GABA-evoked currents in Fig. 3B seem to be smaller in neurons expressing the R952H variant. I suggest the authors simply clarify this point by comparing somatic and dendritic GABA-evoked currents in the different conditions, which should be easy.
With these measurements we aim at obtaining reliable Egaba values and the uncaging intensity is set to provide a good S/N ratio. Therefore, the absolute magnitudes of the currents in the sample traces (Fig 3B) are not comparable. Notably, we have previously shown that changing the laser intensity (thus changing the amplitude of the $GABA_AR$ responses) within the range used under the present conditions presently does not influence the value of E_{GABA} or ΔE_{GABA} (Khirug et al., 2005).

2. The new data convincingly shows the R952H variant causes a defect in KCC2 membrane expression in non-neuronal cells. However, considering the variety of neuron-specific mechanisms (Khale et al TINS 2013) and molecular interactions (4.1N, Neto2, possibly GABAB receptors...) that influence KCC2 membrane traffic, it might be useful to corroborate this biochemical assay with immunocytochemical data in neurons.
As authors of the above TINS review we are not exactly sure what referee 1 wishes to be addressed by 4.1N and Neto2 experiments (data which has been previously shown to be similar in both cell systems and neurons HEK cells (Ivakine et al./Woodin 2013 PNAS; Horn et al./Kaila 2010 EJN; Li et al./Kaila 2007 Neuron). Regarding surface protein analyses, we would like to point out that the most widely accepted data on KCC2 trafficking has been shown using HEK cells (Lee et al./Moss 2007 JBC). We are convinced that our biochemical assay that is sensitive to plasmalemmal protein only provides information that is much more robust than immunocytochemical data.

3. Regarding the dominance of the effect of the R952H variant, as the authors guessed, I meant phenotypic dominance, not genetic. As mentioned in my previous review, although I understand the logic, I question the validity of expressing hKCC2 in immature neurons (which likely do not express much endogenous KCC2 but express higher amounts of NKCC1), to establish the functional impact of the variant (see above).
Under the present conditions with 10 µM bumetanide (which fully blocks NKCC1) our Cl^- extrusion assay is not influenced by NKCC1 activity.

4. I am not competent to fully appreciate or comment on the validity and power of the genetics data. However, in agreement with the other reviewer, I note that rare variants in genes encoding ion transport proteins have a rather poor predictive power on the appearance and severity of epilepsy. A recent study by the Noebels lab (Klassen et al Cell 2011), which I think should be quoted in the present ms., shows that individual variants are just as frequent in patients as in healthy

individuals even though patients more often carry several variants in epilepsy-related genes. Thus, in the absence of exome screening data, identification of a single variant in individuals with a form of epilepsy as frequent as FS may have only a poor predictive power. I believe this point deserves a thorough discussion in the present paper.
The issues concerning the analyses of rare variants and their phenotypic effects have been extensively discussed in the manuscript.

5. Page 4, the authors state that 'the present study provides [...] the first evidence of the role of KCC2 in dendritic spine formation in humans'. This is an intriguing semantic shift, as the present study contains no data on dendritic spines in humans. Instead, what it shows is that WT but not R952H human KCC2 can rescue dendritic spine formation in neurons from KCC2-/- mice. The authors should make sure to amend their text for consistency with experimental data.
We fully agree with the Referee and have therefore deleted this statement.

Referee #2:

From the different approaches the authors used to study the KCC2 variant at the functional level I have no doubt that the variant differs from wild-type. However, whether the variant is disease causing in the family presented is not clear. The family is definitely too small to allow firm conclusions from the segregation analysis. Of course the variant is transmitted to the offspring of carriers with a likelihood of 50%, whether it is disease causing or not. The same holds true for other potentially disease causing variants not identified which may explain / contribute to the phenotype. At best the assumption that the variant identified plays some role for the phenotype appears reasonable. This assumption would be clearly strengthened if the authors could identify a second independent family with febrile seizures co-segregating with this variant. This would be the minimal requirement for a genetic journal.
As explained in our previous response, we don't have any possibility to extend the patient population at a scale which would provide fair chances for detecting another affected family for this very rare variant. Therefore we have down-toned the genotype-phenotype link as instructed by the editors. Please also note that we are not stating R952H to be disease causing, but rather a contributing susceptibility variant.

One also has to bear in mind that the experimental design in this manuscript does not reflect the situation in the family presented, in which the variant protein is co-expressed with wild-type. Is it known whether heterozygous knockout neurons show alterations in spine morphology? If not, I would not expect the latter in heterozygous carriers of the variant, unless one would claim a dominant negative effect, which is difficult to imagine.
A gene dose effect on dendritic spine morphology is clearly seen in KCC2 hypomorphic mice (Li et al./Kaila 2007)

The new set of data addressing expression and plasma membrane targeting of the variant is a very important additional piece of information. For the interpretation it would be nice if to state whether the cell line expresses KCC2 endogenously. The targeting defect may be even stronger, if the antibodies used cross react with both the human and the mouse orthologue.
In line with their low level of differentiation, cells in clonal lines do not express endogenous KCC2 (see for instance Horn et al, 2010 EJN).

1st Editorial Decision 10 March 2014

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