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α-Actinin-1 promotes activity of the L-type Ca^{2+} Channel Cav1.2

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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.)

1st Editorial Decision 5th Jul 2019

Thank you for the submission of your manuscript (EMBOJ-2019-102622) to The EMBO Journal. Your manuscript has been sent to three reviewers, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your results, although they also express a number of issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail, referee #3 states a number of conceptual concerns about your proposed model and conclusions and asks you to complement the study with additional experiments to consolidate physiological relevance of your findings with additional experiments in neuronal cells (ref#3, pt.2), explore potential synergistic interplay between Cav1.2 IQ domain with ApoCaM and alpha-actinin in a trimeric complex (ref#3, pt.1, see also ref#1's point on binding mutants), and test the cation type-dependence of the results (ref#3, pt.3). In addition, the reviewers raise a number of issues related to methods annotation, statistics, appropriate discussion of the results as well as wording that would need to be conclusively addressed to achieve the level of robustness and clarity needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. We do concur with the reviewers that in light of the findings contrasting earlier work in the field, excluding potential ambiguities and providing results of high robustness will be essential.

REFEREE REPORTS:

Referee #1:

L-type voltage-gated Ca2+ channels are key regulators of cardiovascular, endocrine, and neuronal function. This manuscript by Turner et al. reports several important new findings that significantly modify and extend our understanding regarding how L-type voltage-gated Ca2+ channels are regulated by molecular interactions with the apo-form of the EF-hand Ca2+ binding protein calmodulin (CaM) and the EF-hand domains of the actin cytoskeleton-associated scaffold protein alpha-actinin. The authors employ a powerful combination of NMR-spectroscopy structure determination, biochemical assays of protein binding, and single-channel electrophysiological recordings to reach the conclusion that competitive binding of alpha-actinin to the Cav1.2 IQ CaM binding domain not only regulates L-channel targeting to the cell surface, as previously appreciated, but also directly increases channel open probability (Po) by promoting coupling of gating charge movement to channel opening. Additional data presented also indicates that the affinity of the Cav1.2 IQ domain for apo-CaM is much lower under physiological salt concentrations conditions than previously thought, suggesting that apo-CaM does not likely basally interact with Cav1.2 under physiological conditions in cells such as neurons. Accordingly, the authors go on to show that apo-CaM binding to the Cav1.2 IQ domain is not required for maintaining basal channel open probability, which is in contrast to the conclusions of previous high-impact published work from David Yue's group (Adams et al., 2014 Cell) that relied on an I to A mutant in the IQ domain to disrupt apo-CaM binding. Importantly, the authors here show that this IA mutant not only disrupts apo-CaM biding to the Cav1.2 IQ domain but also disrupts alpha-actinin binding. These new findings regarding how alpha-actinin, but not apo-CaM, binding controls L-channel Po indicate that current mechanistic models for how CaM regulates L-channel function during Ca2+-dependent dependent inactivation (CDI), which acts as an important negative feedback mechanism to limit Ca2+ entry through these channels, should be re-evaluated.

Key findings reported by this study are: 1) two novel NMR solution structures of apo-CaM and the alpha-actinin EF3,4 domain bound to the Cav1.2 IQ motif; 3) structural identification and functional validation by mutagenesis and binding assays of key residues in the Cav1.2 IQ domain that either regulate both apo-CaM and alpha-actinin binding (I1654A) or only alpha-actinin (K1647E, Y1649A) or apo-CaM (F1658A, K1662E) binding specifically; 4) IQ domain mutations in residues that specifically impair alpha-actinin binding decrease channel Po in single channel recordings, while mutations that specifically impair apo-CaM binding have no impact; 5) alpha-actinin binding to the Cav1.2 IQ domain increases Po by increasing coupling of gating charge movement to channel opening. Importantly, the conclusions of these biochemical and electrophysiological experiments are all reinforced by additional mutagenesis of key corresponding binding residues in alpha-actinin or CaM, including an elegant "charge reversal" rescue.

Overall this study is very comprehensive and carefully executed. The data are of high quality and the findings are important. I have no major concerns, but do have one minor concern that should be addressed regarding how the new findings may change how we should think about CDI. At minimum, the authors should at least further discuss how alpha-actinin regulation of Po and CDI may be connected. In particular, CDI requires Ca2+-CaM binding to the IQ motif, which also displaces alpha-actinin binding as previously shown by the authors in Tseng et al 2017. In addition, the authors mention in the Discussion that alpha-actinin binding increases Po by promoting coupling of gating charge movement to channel opening like previously shown for PKA regulation (Fuller et al, 2010). A requirement for alpha-actinin binding to increase Po as a prerequisite for subsequent CDI would further parallel PKA regulation of current density and Po that is intertwined with priming channels to under subsequent CDI mediated by Ca2+-CaM binding and reversal of PKA actions via dephosphorylation by the phosphatase PP2B. (See Chad and Eckert, 1986; Armstrong and Eckert, 1987; Oliveria et al., 2007,2012; Dittmer et al. 2012).

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Using NMR to solve structures of CaV1.2 α1 subunit bound to either α-actinin-1 and to apo-calmodulin in combination with patch clamp electrophysiology and surface biotinylation assays in 293 cells, Turner et al studied how CaV1.2 activity is regulated by these by these interactions and builds upon their previous study Tseng et al 2017 PMID:28613835 that identified that the α-actinin-1 was important for membrane localization. In this study the solved NMR solutions structures give insight to the how the EF hand region of α-actinin-1 and apo-calmodulin interact with CaV1.2 IQ motif. Subsequent, characterization demonstrate that the α-actinin-1 not only impact CaV1.2 surface localization as previous reported, by Tseng et al 2017, but they find that the α-actinin-1 interactions are positive regulators CaV1.2 channel open probability and gating. Importantly they show that apo-calmodulin interaction does not regulate CaV1.2 channel open probability and gating. These findings demonstrate that while α-actinin-1 and apo-calmodulin both interact with the IQ motif the respective interactions differentially regulate CaV1.2 channels and that apo-calmodulin regulation of CaV 1.2 is distinct from CaV 1.3. Based on these findings their main conclusion is that the α-actinin-1 regulation of CaV1.2 channel activity ensures that CaV1.2 channel activity is minimal while trafficking to its proper location at the cell surface.

Overall the experiments in this study are very well designed and executed, analyses are quite thorough. The electrophysiological data elegantly integrates the findings of the NMR solution structures and offers potential mechanistic insights into understanding how Cav1.2 channel activity is regulated outside of its proper location. The findings in this study are important for many cell types that utilize CaV1.2 channels and therefore of general significance for understanding the mechanisms of CaV1.2 channel regulation.

Major comments.
1) Eleven supplemental figures are excessive, and some supplemental figures appear to just be different graphs of the same data found in the main figures. To give a better representation of the data and make it easier on the reader the authors should present data either as box mean plots, or scatter plots in the main text and get rid of the duplicative supplemental data. Also, figure 4 And 5 could be consolidated.

2) Figure 5. For the experiments analyzing of EE/KK the control there is 2 fold more control than mutant. The scatter plots in the supplemental data give the appearance of a different distribution between the two data sets although the means appear to be similar. I am not arguing for P hunting but having 2X more data collected for the WT compared to the EE/KK data will affect the robustness of the statistical test. I highly recommend that the authors increase the sample numbers for this experiment. This will allow them to draw a firm conclusion.

3) The discussion highlights the Nav1.2 structure compared their findings. Since this is a major point of their discussion they should move this supplemental figure into the main text. This makes it easier on the reader if this figure is in the main text.

Referee #3:

In this manuscript Turner and his colleagues examine the molecular mechanism underlying upregulation of Cav1.2 activity by alpha-actinin-1. In previous work the same group had shown that alpha-actinin-1 binds to the IQ motif of Cav1.2. Furthermore, they had identified three key residues in the sequence of the IQ motif that were crucial for this interaction. In the present study, NMR is employed to determine the 3D structure of the Cav1.2 IQ bound to the two distal EF hands (EF3/4) of alpha-actinin-1. The authors identify three charged amino acid residues (K1647 in IQ and E847/E851 in alpha-actinin-1) that form salt bridges between the IQ and EF3/4, and a hydrophobic interaction (I1654 in IQ, F833 in alpha-actinin-1) as crucial determinants for the interaction. Mutation of the charged residues abolished interaction while binding could be rescued by charge inversion mutagenesis. In a series of cell-attached single channel patch -clamp experiments the

authors show that alpha-actinin-1 increases the open probability (Po) of Cav1.2. In agreement with their NMR studies and binding assays effects on Po were strongly dependent on the identified charged amino acid residues. Charge movement measurements suggest that the stimulatory effect on Po is caused by an augmentation of gating charge movement including downstream coupling of charge movement to pore opening. Finally, the authors have also determined a NMR structure of the apocalmodulin (apoCaM) / Cav1.2-IQ complex. They show that the apoCaM binding site is distinct from that of alpha-actinin-1. Surprisingly, apoCaM did not affect Po of Cav1.2. This finding contrasts with previous work of the Yue group on the related Cav1.3 channel (Adams et al. Cell 2014) that proposed an increase of Po induced by apoCaM.

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1. In native cells (e.g. neurons) Cav1.2 is coassembled with both alpha-actinin-1 and apoCaM. Since both proteins can bind to the IQ they may exert complex (e.g. synergistic) functional effects on Cav1.2 that are not seen when the channel is only characterized in the presence of alpha-actinin-1 or apoCaM alone. For example, the authors discuss that the conclusion of the Yue group that apoCaM enhances Po could reflect the fact that I1655 in the IQ not only contributes to apoCaM but also to alpha-actinin-1 binding. Given this complexity, I feel that it is necessary to examine interactions between all three components in a trimeric complex (IQ + apoCaM + alpha-actinin-1) in binding assays, NMR and patch-clamp.

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1st Revision - authors' response 13th Nov 2019

Referee #1:

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suggesting that apo-CaM does not likely basally interact with Cav1.2 under physiological conditions in cells such as neurons. Accordingly, the authors go on to show that apo-CaM binding to the Cav1.2 IQ domain is not required for maintaining basal channel open probability, which is in contrast to the conclusions of previous high-impact published work from David Yue's group (Adams et al., 2014 Cell) that relied on an I to A mutant in the IQ domain to disrupt apo-CaM binding. Importantly, the authors here show that this IA mutant not only disrupts apo-CaM biding to the Cav1.2 IQ domain but also disrupts alpha-actinin binding. These new findings regarding how alpha-actinin, but not apo-CaM, binding controls L-channel Po indicate that current mechanistic models for how CaM regulates L-channel function during Ca2+-dependent inactivation (CDI), which acts as an important negative feedback mechanism to limit Ca2+ entry through these channels, should be re-evaluated.

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The notion that alpha-actinin binding to increase Po might be a prerequisite for subsequent CDI, with CDI possibly being at least in part accomplished by displacement of alpha-actinin by Ca/CaM, is definitely highly intriguing. It would be analogous to the antagonistic roles PKA and the Ca/CaM-activated phosphatase calcineurin/PP2B play, with PKA augmenting Po and thereby priming Cav1.2 for CDI, and Ca/CaM activating calcineurin to dephosphorylate Cav1.2 at its PKA site(s). We spent a substantial amount of time and effort to evaluate this possibility over the last few months (Fig. A). However, our data suggest that CDI is actually normal for Y1649A mutant Cav1.2 in which alpha-actinin and not CaM binding is affected (Fig. A). As a positive control CDI is impaired for the 'classic' I1654A mutation (Fig. A) as shown earlier (Zühlke et al., 1999: Nature 399, 159-162; Zühlke et al., 2000: J Biol Chem 275, 21121-21129). This loss of CDI is likely because I1654 is central to binding of Ca/CaM (Van Petegem, Chatelain, and Minor, 2005: Nature Struct Mol Biol 12, 1108-1115; Fallon et al., 2005: Structure 13, 1881-1886), which is required for CDI (Peterson et al., 1999: Neuron 22, 549-558). Thus, against the initial captivating hypothesis here, we do **not** believe that CDI is due to a reduction of Po as a result of an immediate (within ms) displacement of alpha-actinin even though binding of Ca/CaM is required for CDI. The lack of CDI for the I1654A mutant is thus most likely due to loss of CaM binding but not alpha-actinin binding.

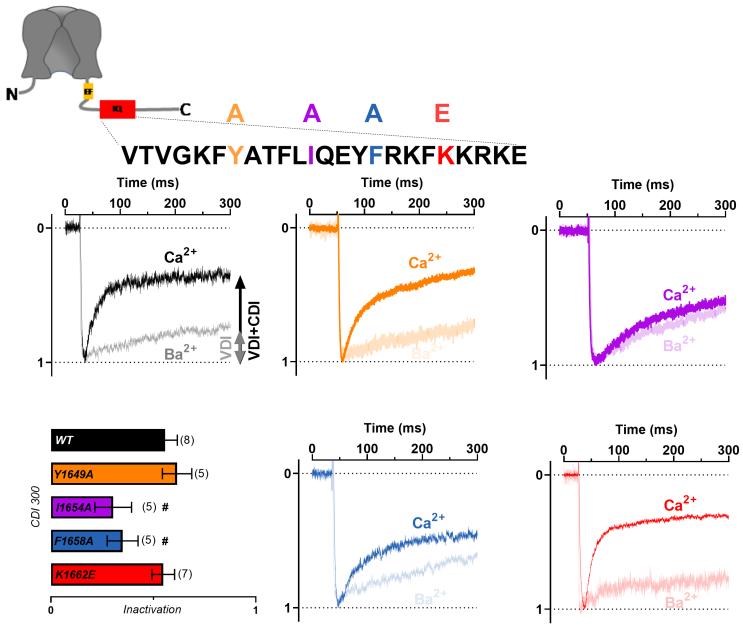

Fig. A: CDI analysis. HEK293 cells were transfected with $\alpha_1$1.2, $\alpha_2\delta$-1, and β_{2A} before whole cell patch recording in 20 mM Ba^{2+} (light traces) or 10 mM Ca^{2+} (dark traces) for WT (gray/black), Y1649A (orange), I1654A (purple), F1658A (blue) and K1662E (red). Shown are representative current traces of the first 300 ms obtained from recordings upon depolarizations from a holding potential of -80 mV to +20 mV. Bar diagram shows **CDI** as the fractional difference between the residual current at 300 ms (R_{300}) in 10 mM Ca^{2+} versus R_{300} in 20 mM Ba^{2+} (50-60% for WT, Y1649A, K1662E and ~35% for I1654A and F1658A; n=5-8; # <0.05, unpaired T-test).

We also analyzed the effects of our mutants that impair apoCaM binding on CDI. We find that **CDI of Cav1.2 K1662E is actually not different from CDI for WT** (Fig. A). This finding is consistent with earlier results by the late David Yue and

co-workers in which the equivalent mutation in Cav1.3 did not affect CDI (Ben-Johny et al., 2013: Nat Commun 4, 1717, Fig. 3; Bazzazi et al., 2013: Cell Reports 5, 367-377, Fig. 2). This result actually suggests that pre-association of **apo**CaM with the Cav1.2 IQ motif is **not** required for CDI because K1662 forms a strong salt bridge with **apo**CaM (Fig. 2 in our manuscript) and the Kd value is increased by 6-fold (i.e., affinity is decreased) for the K1662E IQ peptide versus WT IQ peptide (Table 4). At the same time K1662 makes minimal contact with **Ca**/CaM in the crystal structure (Van Petegem, Chatelain, and Minor, 2005: Nature Struct Mol Biol 12, 1108-1115; Fallon et al., 2005: Structure 13, 1881-1886). These findings are consistent with the model we propose in the Discussion that the main species of CaM associated with Cav1.2 under resting conditions is CaM with Ca^{2+} bound to EF3 and EF4 in the C lobe (please see Discussion for more details). In further support of this model, F1658 forms several interactions with residues of Ca/CaM and thus should impair CDI. In fact, such an impairment was reported for the equivalent mutation in Cav1.3 (Ben-Johny et al., 2013: Nat Commun 4, 1717, Fig. 3; Bazzazi et al., 2013: Cell Reports 5, 367-377, Fig. 2). Accordingly, we find that the **F1658A** mutant Cav1.2 has **decreased CDI vs. WT** (Fig. A). However, we are performing further structural and functional analyses to thorough address the question of whether pre-association of Ca/CaM with the IQ motif is required for CDI before we feel we can publish in detail and with greater depth on this issue. These experiments, which include new structural analyses, will take much more time than can be accomplished in the next few months and thus have to remain beyond the scope of our already very extensive work on how alpha-actinin affects Cav1.2 function under basal conditions.

Referee #2:

Using NMR to solve structures of CaV1.2 α1 subunit bound to either α-actinin-1 and to apo-calmodulin in combination with patch clamp electrophysiology and surface biotinylation assays in 293 cells, Turner et al studied how CaV1.2 activity is regulated by these by these interactions and builds upon their previous study Tseng et al 2017 PMID:28613835 that identified that the α-actinin-1 was important for membrane localization. In this study the solved NMR solutions structures give insight to the how the EF hand region of α-actinin-1 and apo-calmodulin interact with CaV1.2 IQ motif. Subsequent, characterization demonstrate that the α-actinin-1 not only impact CaV1.2 surface localization as previous reported, by Tseng et al 2017, but they find that the α-actinin-1 interactions are positive regulators CaV1.2 channel open probability and gating. Importantly they show that apo-calmodulin interaction does not regulate CaV1.2 channel open probability and gating. These findings demonstrate that while α-actinin-1 and apo-calmodulin both interact with the IQ motif the respective interactions differentially regulate CaV1.2 channels and that apo-calmodulin regulation of CaV 1.2 is distinct from CaV 1.3. Based on these findings their main conclusion is that the α-actinin-1 regulation of CaV1.2 channel activity ensures that CaV1.2 channel activity is minimal while trafficking to its proper location at the cell surface.

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Major comments.
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We moved the dot plot diagrams to the main text to eliminate those from the Supplemental Data section. We reduced the number of sample sweeps we show in Fig 3, 4, and 5 but show all original sample sweeps as Expanded View figures.

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We completely agree that it would have been reassuring if we could have performed more of these recordings and obtain p values that would perfectly match the criterion for rejecting the null hypothesis (i.e., $p<0.05$). However, we had to make a choice in how to allocate our efforts and especially the time the electrophysiologist in my lab would spend on the different requests by all Reviewers. We spent a lot of time on testing whether binding of alpha-actinin to the IQ motif is a prerequisite for CDI and whether displacement of alpha-actinin by Ca/CaM could drive CDI, as suggested by Reviewer 1. We also extensively tried to get recordings from neurons, which, unfortunately, did not pan out (see Reviewer 3 #2).

3) The discussion highlights the Nav1.2 structure compared their findings. Since this is a major point of their discussion they should move this supplemental figure into the main text. This makes it easier on the reader if this figure is in the main text.

We moved this Figure to Expanded View, which is immediately accessible through the EMBO J portal.

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IQ and EF3/4, and a hydrophobic interaction (I1654 in IQ, F833 in alpha-actinin-1) as crucial determinants for the interaction. Mutation of the charged residues abolished interaction while binding could be rescued by charge inversion mutagenesis. In a series of cell-attached single channel patch -clamp experiments the authors show that alpha-actinin-1 increases the open probability (Po) of Cav1.2. In agreement with their NMR studies and binding assays effects on Po were strongly dependent on the identified charged amino acid residues. Charge movement measurements suggest that the stimulatory effect on Po is caused by an augmentation of gating charge movement including downstream coupling of charge movement to pore opening. Finally, the authors have also determined a NMR structure of the apocalmodulin (apoCaM) / Cav1.2-IQ complex. They show that the apoCaM binding site is distinct from that of alpha-actinin-1. Surprisingly, apoCaM did not affect Po of Cav1.2. This finding contrasts with previous work of the Yue group on the related Cav1.3 channel (Adams et al. Cell 2014) that proposed an increase of Po induced by apoCaM.

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We conducted multiple NMR titrations to probe structural aspects of the hypothesized ternary complex (Expanded View Fig. 3). However, all results were negative and we conclude that the IQ peptide, apoCaM, and alpha-actinin-1 cannot form a ternary complex. We provide the NMR spectra in Expanded View Fig. 3 and describe these spectra in a new section in the Results on page 10 (lines 212-227). The lack of a ternary complex is consistent with our finding that Ile1654 forms multiple contacts with both, alpha-actinin-1 EF hand region and apoCaM and that Ile1654 is required for both alpha-actinin-1 binding and apoCaM binding. Thus, binding of apoCaM and alpha-actinin-1 to the IQ motif at least as far as it involves the eponymous Ile should be competitive and occlude binding of the other partner. Our new NMR data (Suppl Fig. 12) now demonstrate that apoCaM and alpha-actinin bind competitively to the IQ motif and the IQ peptide does not bind simultaneously to both apoCaM and alpha-actinin.

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The question how Cav1.2 interacts with alpha-actinin and CaM in a native system is definitely an important one. We mutated T1066 of Cav1.2 to Tyr, which makes Cav1.2 dihydropyridine-insensitive. We express T1066Y Cav1.2 in our high quality cultured hippocampal neurons. Application of the dihydropyridine isradipine does block all endogenous L-type currents in the absence of ectopically expressed T1066Y Cav1.2. However, and against expectation, even with ectopic expression of Cav1.2 T1066Y isradipine abrogated nearly all activity in our single channel recordings. We re-sequenced Cav1.2 to make sure that the mutation was present. Despite extensive trouble shooting and multiple attempts we were not able to get this strategy to work.

3. It is obvious that Cav1.2 currents are much easier to characterize using Ba2+ instead of Ca2+ as charge carrier. Nevertheless, physiologically the channels conduct Ca2+. Since the IQ motif is involved in calcium-dependent inactivation which may profoundly interfere with effects reported in this study the authors should complement their analysis with electrophysiological data using Ca2+ as charge carrier. These experiments are crucial to demonstrate that proposed alpha-actinin-1 effects are independent of the cation passing the channel.

We tried very hard to record single channel activity in HEK293 cells with Ca rather than Ba as charge carrier. However, Ca provides much smaller currents and we were not able to discern those from the noise. Our initial attempts with 1.8 mM and 10 mM Ca under our standard cell attached recording conditions did not yield any discernable currents above our noise level, which is roughly 0.4 pA. We then performed experiments using borosilicate glass with an outer diameter of 1.5 mm and inner diameter of 0.86 mm and heavy elastomer coating (Levis and Rae 1993: Biophys J 65, 1666-1677). For that, Sylgard was applied close to the tip of the recording pipette to improve the seal and thereby reduce noise. However, this strategy still did not improve the noise level sufficiently enough for detection of unitary Ca currents.

Thus, finally, we resorted to whole cell patch recordings with Ca to confirm the effect we saw with Ba. We found an ~80% reduction in whole cell current for the key K1647A mutation, the only mutation we tested due to time restraints, versus WT Cav1.2.

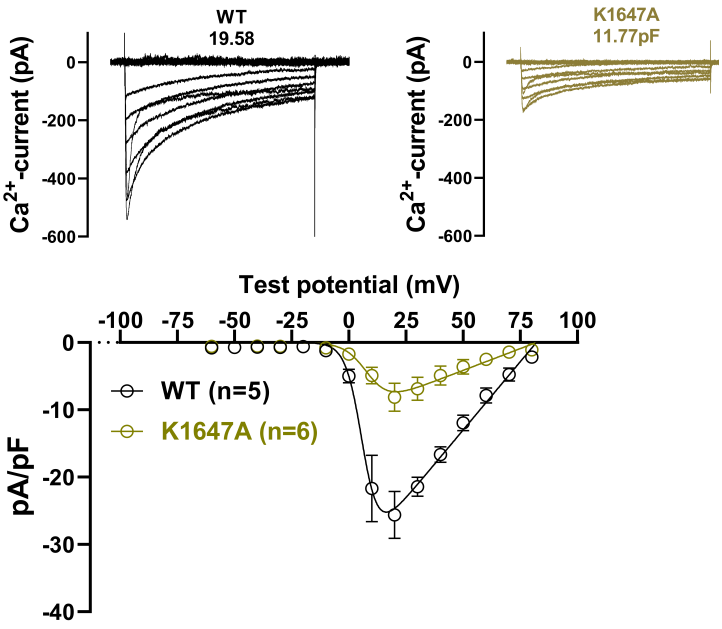

Fig. B: Analysis of whole cell currents with Ca as charge carrier. HEK293 cells were transfected with $\alpha_1$1.2, $\alpha_2\delta$-1, and β_{2A} before whole cell patch recording in 10 mM Ca^{2+} from a holding potential of -70 mV to the indicated potentials. **Top** shows sample recordings to the various test potentials for WT Cav1.2 (left) and K1647A mutant Cav1.2 (right). **Bottom** shows I/V curves from 5-6 experiments.

2nd Editorial Decision 26th Nov 2019

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your amended study was sent back to two of the referees for re-evaluation, and we have received comments from both of them, which I enclose below.

As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues related to formatting and data representation as listed below, which need to be adjusted at re-submission.

REFEREE REPORTS:

Referee #2:

The authors have adequately addressed my previous comments. Congrats to the authors on a great study.

Referee
#3:

The authors have addressed all three points raised in my previous review. Importantly, their new NMR data convincingly demonstrate that apoCaM and alpha-actinin bind competitively to the IQ motif of Cav1.2 rather than forming a ternary complex. The authors also show macroscopic Ca2+ currents of the K1647A mutant that support the basic conclusions of this study. Unfortunately, due to technical problems, the author failed to characterize Cav1.2 in hippocampal neurons. Although it would be nice to have such data I don't think that they are absolutely crucial. In summary, the revision has profoundly improved this manuscript. I have no further objections.

2nd Revision - authors' response 4th Dec 2019

The authors have performed the requested editorial changes.

3rd Editorial Decision 6th Dec 2019

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Johannes W. Hell
Journal Submitted to: EMBO
Manuscript Number: EMBOJ-2019-102622R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample sizes for our biochemical, electrophysiological and immunofluorescence analyses was chosen basedon extensive previously published work indicating that sample sizes were adequate.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Single channel data were only analyzed for n<3 channels in the patch to not overstate channel open probability. See also Herzig et al., 2007 and Bartels et al., 2018
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Single channel recordings and Popen analysis were performed based on a single blinded approach. In brief, transfected cells were handed over to the patcher only with a code which was only reveiled after data analysis (Popen).
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The data population distribution was tested by a D'agostino & Pearson Test. The data showed a normal or log-normal distribution and were further tested for significance with an ANOVA with Bonferroni correction. See also Material and Methods.

Is there an estimate of variation within each group of data?	Standard error of mean (SEM) is depicted for all data.
Is the variance similar between the groups that are being statistically compared?	The variance between the groups is similar due to similar sample size between groups

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	See "Materials and Methods"
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK fromcells a standard source were used in our studies which were in the past tested negative for mycoplasm contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The NMR assignments have been deposited in the BMRB (accession number 25902). The atomic coordinates have been deposited into the Protein Databank (6COA and 6CTB).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA