

Altered axonal initial segment development links circuit and Kv7 dysfunction in an *Fmr1* knockout rat.

Corresponding Author: Professor Michael Cousin

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript shows developmentally transient alteration of intrinsic excitability and presynaptic function in CA1 pyramidal neurons from *Fmr1* knockout, a rat model of fragile X syndrome (FXS) at P12–15, associated with loss of voltage-gated potassium KCNQ channel (Kv7) function. Redistribution of Kv7 channel in the spike-generating compartment within the axonal initial segment in *Fmr1* knockout neurons was found.

Intrinsic neuronal properties and baseline neurotransmission evoked via a single stimulus were shown to be unaffected in male *Fmr1* KO rats throughout development. However, in P12-15 *Fmr1* KO rats there was a trend towards an increase in total current transfer over the course of stimulation, suggesting increased neurotransmission. A greater synaptic vesicle replenishment rate and a trend towards an increased readily releasable pool size in *Fmr1* KO synapses compared to WT were shown, suggesting an enhancement in the replenishment of the recycling vesicle pool in *Fmr1* KO animals. The findings were not conserved in 6-10-week-old rats. Alterations in P12-15 *Fmr1* KO rats were linked to dysfunction of Kv7 channels based on previous studies in mice and effects of retigabine were studied. The Kv7 agonists did not have significant impact on action potential dynamics in *Fmr1* KO synapses and it was found that Kv7 channels do appear to be capable of modulating replenishment of the SV recycling pool, but only during lower intensity stimulation. Finally, changes in AIS morphology were shown to underpin alterations in Kv7.2 distribution, potentially causing FXS-specific changes.

The data are interesting and confirm the previously shown contribution of Kv7 channels to the pathophysiology of FXS. The results are generated using sufficient number of animals and although the findings are often marginal with high variation, they are well described. The discussion remains limited related to the potential mechanisms underlying differences observed between mouse and rat. The manuscript is carefully written and the text is fluent (only in Results one misspelling appeared as "in" twice). Based on the previous studies and the interesting new AIS results, the differences obtained may have impact and particularly affect the developmental trajectories, considered as the main message of the manuscript. The following issues remain to be addressed:

- Since the electrophysiological properties can be region- and cell-type dependent (Brandalise et al., 2020; Kalmbach et al., 2015) and Kv7 channels are widely expressed in neurons, it would be important to determine Kv7 expression in the rat FXS model as done in *Fmr1* KO mice.
- In results text needs to be revised to study "conserved abnormalities P6-10 week".
- Is the high variation in results at P12-15 caused by developmental change and could more accurately determining the day improve results? Another developmental time point for the studies would be needed.
- The differences between mouse and rat have to be better explored.
- In figure legend N/n values are given and although it is indicated in Materials and methods, it would be good to indicate it at least in the first figure legend, too.

Reviewer #2

(Remarks to the Author)

In this study, Webster et al examine a potential role for Kv7 channels in excitability alterations in a rat model of Fragile X Syndrome, the *Fmr1* knockout (KO). Importantly, they find changes in juvenile animals at P12-15 that have recovered by adult stages. Careful electrophysiological experiments define changes to repetitive action potential (AP) firing, with decreased firing rates at high levels of current injection paralleled by progressive widening of AP width (relative to the start

of injection). In addition, they find altered handling of synaptic vesicle pools, with a faster replenishment rate in the Fmr1 KO neurons. To various extents, these alterations seem to be at least partially mediated by dysfunction in Kv7 potassium channels. Overall, this is a very well carried out study which adds to the growing literature on excitability changes and indeed the part played by potassium channels in models of Fragile X syndrome. I have several minor issues which could be addressed by the authors.

Some of the results are quite confusing, which is rightly acknowledged by the authors in the discussion. The data seem to point to dysfunction of Kv7 channels, but this only seems to be reflected in a wider AP (not a usual feature of Kv7 loss of function) without any of the expected effects of decreased Kv7 function, such as a more depolarized resting membrane potential, decreased AP threshold etc, and indeed one would predict increased firing rates, but in fact they find the opposite – that firing rates are decreased (albeit at high current injections). Even more strangely, while Kv7 blockade has the predicted effects on both genotypes, indicating that Kv7 channels are present and active, Kv7 activation has no effect on the Fmr1 KO neurons. I am not sure I am convinced by the explanation provided that perhaps there is functional decoupling – if this were the case, you might expect that blockade would have no effects. While I believe the data 'is what it is' and I suspect there may be something more complex and interesting underlying this, a key experiment that would be worth checking is to measure the M current itself (WT vs KO, as well as after Kv7 agonism/antagonism). This would also be a much better way to assess what has happened to Kv7 function versus immunostaining which is notoriously difficult to quantify and see differences with. It would also be very helpful if the authors could include the data showing the effects or not of Kv7 activation / blockade on RMP and AP threshold. Finally, please add baseline, actual, single AP width data – while this is not expected to be changed, it is important given the later results.

The authors examine AIS morphology in dissociated cultures, citing 'similar.. defects' from their previous work. However, this does not examine excitability at all, and there can be big differences between dissociated culture and more intact preparations at this level. I would suggest that it should be straightforward to analyse AIS morphology at least in sections of the hippocampus, which would add much weight to these conclusions, and it would be worth trying to also image Kv7.2, although I appreciate this might be more difficult.

For the experiments with the Kv7 agonists/antagonists, although the WT and KO firing rate and AP width graphs are not overlaid, I wonder if there are still significant changes seen (in the no drug controls)? If not, this might account for some of the surprising findings. It might also be as a result of variability (likely higher in rats) and perhaps these are not littermate experiments, but if so, this could be mentioned.

The main text is not in the same order as the figure panels, which makes it somewhat confusing for the reader. I recommend matching them. Personally, I also think it would be easier to discuss first the retigabine experiments and then XE991.

Appropriate sample sizes are used throughout, but please make it clear in the first figure legend what the notation means – I assume it is cells/animals, but this should be added.

P6, 1st paragraph, line 4. 'In agreement...' – would be good to rephrase so that it is clear that this is referring to previous work. Eg 'previous work in mice has shown....'

P7, para 4. 'The rationale being that roscovitine...' - this is not a sentence.

P13, line 1 – no apostrophe after its.

Reviewer #3

(Remarks to the Author)

The authors set out to characterize intrinsic excitability of CA1 pyramidal neurons and presynaptic properties in a Fmr1 KO rat model of Fragile X syndrome with a particular focus on two developmental time windows. Key findings include an action potential broadening and enhanced SV pool replenishment observed in P12-15 Fmr1 KO rats which both do not persist into adolescence. Furthermore, they propose a potential link to altered Kv7 channel function based on axon initial segment remodelling and channel redistribution.

The paper is very interesting, however several points should be addressed and clarified, please.

Were the recordings performed in dorsal or ventral hippocampus?

The terminology "loss of Kv 7 function" is confusing to me since usually reduced Kv7 function is associated with increased excitability and not reduced excitability as presented here.

Fig. 1:

Can you please speculate why rheobase and input resistance are unchanged despite the strong trend to hypoexcitability of KO CA1 pyramidal neurons?

Fig. 2, Fig. 4 and Fig. 5:

The authors link hypoexcitability shown in Fig. 2 to an altered function of Kv7 channels. Hypoexcitability would be compatible with an increased Kv7 function. Therefore, in my mind the next logical experiment would have been a rescue attempt with XE991 to check whether the hypoexcitability can be reverted. Whereas the authors interpret the data shown in

Fig. 5 A and B as an indication of enhanced excitability due to left-shifted input-output curves in presence of XE991, I actually rather had the impression that the number of fired APs further declined in presence of XE991 for current injections above 250 pA and remained basically unchanged or only marginally increased for smaller injections. Also, according to the statistical tests data only showed a trend. An important point might be that hypoexcitability for the KO in comparison to the wildtype emerges only for current injections of 300 pA and more, when neurons increasingly start to plateau. XE991 seems to amplify this effect due to Kv7 blockage, associated reduction of M-current and reduced spike-frequency-adaptation in combination with electrophysiological immaturity (in contrast to the juvenile time point at which current injections up to 500 pA are tolerated without AP breakdown/plateauing). Can you please clarify this.

Expectedly, retigabine decreased the AP firing in the wildtype condition (Fig. 4B) but it also affected AP firing in the KO. This effect occurred primarily for current injections below 300 pA and was absent for higher current injections (thereby all in all displaying a different pattern than the one with XE991 and also a different pattern than the one seen when comparing KO and wildtype). I agree that the retigabine effect is less pronounced in KO neurons. Yet, XE991 appears to cause comparable effects in wildtype and in KO. Taken together, I feel that additional features such as altered mAHP and spike-frequency-adaptation in KO versus wildtype could help to substantiate the role of Kv7. Did you attempt to analyze these parameters? On the other side, M-current recordings (could be estimated by recordings before and after XE991) would provide a more direct confirmation of a role of Kv7 channels. In case M-current was indeed reduced at P12-15 it would be interesting to see whether M-current normalizes in the juvenile time window.

Furthermore, whereas XE991 broadened the APs in wildtype and KO neurons, retigabine did not rescue the AP half width of KO neurons. I value the effort the authors took to investigate the distribution of Kv7 channels at axons and specifically at the AIS. However, given that a role of BK channels was previously demonstrated in AP broadening (in CA3 of Fmr KO mice) I find it hard – based on the presented data, particularly in absence of any BK channel testing and M-current recordings – to conclude that in the rat model reduced Kv7 function represents a major correlate. I feel the study would benefit from an experiment using BK channel modulators to demonstrate that BK channels are actually not the main contributors in the rat model. Was the fAHP actually comparable between wildtype and KO?

Table S1 shows that the AP half-width is unchanged between wildtype and KO. Were these values derived from the first AP or from the 5th one? Which current step was used for the analysis?

Given the importance of the retigabine experiments, I wonder whether the authors initially tested different concentrations? 2.5 μ M may have been a bit on the low side.

Did I get this right that retigabine is thought to also have a direct effect in the presynaptic compartment/on presynaptic glutamatergic function, dissociated from its effect on AP firing (soma/AIS)? The synaptic effect seems to rather align with an increase of excitability, which would be an undesired effect for an antiseizure drug. Can you please comment on this, since this may have broader implications.

Aside from the axon/AIS staining Fig. 6 also shows strong somatodendritic Kv7 signal. Were there any obvious differences between KO and wildtype?

In Fig 4Bi (upper left panel, DMSO wildtype) the box around the APs appears to be misplaced.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised manuscript includes many new data and the difference between mouse and rat data is better discussed. However, the mechanisms underlining the species-specific difference in the results remain open. The developmental stage-dependent changes are not fully sufficiently justified; input resistance shown in the figure provides actually some evidence of reduced KO values at P15.

Before publication careful proofreading would be needed i.e. line 80, removed the repeated "in."

Abbreviations in Methods should be consistent (SI); minutes (line 98, 141, 197, 295), seconds (line 161), hours (line 214,

219, 306).
20% line 122
Line 171 , destruction of the brain?
Line using "free-floating method" for clarity.
Consistent use % without space
Line 252, not recommended to start a sentence with numbers.

Reviewer #2

(Remarks to the Author)

The authors have conducted several new experiments and have addressed all my concerns satisfactorily. I just have two tiny comments:

Abstract and line 55 – should spell out that 'P12' means postnatal day 12.

The explanation given to reviewer 3 about neurons not being hypoexcitable but rather entering depolarization block is helpful, and if there is a way to fit it into the discussion I think will be very helpful for readers, as the data remain a little confusing and surprising for people who usually think of Kv7 loss leading to hyperexcitability.

Reviewer #3

(Remarks to the Author)

Thank you to the authors for thoroughly addressing my comments.

The authors took great effort by adding entirely new data and considerably revising their manuscript. Hypotheses and conclusions were markedly strengthened.

I would like to point out that M-current recordings were performed as suggested and are displayed now in new Figure 7. In line with the authors' hypothesis of a functional decoupling of Kv7 channels due to altered properties of the AIS M-currents were shown functionally intact.

Furthermore, additional parameters I had asked for (including mAHP and spike-frequency-adaptation) are now provided for all conditions in supplemental tables.

Thank you for providing additional data using a BK channel activator along with an analysis of fAHP in wildtype and Fmr1 KO indicating that BK channels are less of a contributing factor to dysfunction in the rat than in the mouse model. Additional sentences on this observation were added to the Discussion.

The presented immunocytochemical Kv7 labeling demonstrated somatodendritic signal on top of the AIS labeling. The authors now added an analysis also of the somatodendritic compartment both of cultured neurons and in fixed slices, further substantiating that the main alterations are occurring at the AIS.

I have no further questions.

I noticed two small typos:

Page 17, line 512: "was unchanged in Fmr1 KO synapses" -> "synapses" probably needs to be deleted

Page 22, line 665: "total gross Kv7.2 expression in unaltered" -> is unaltered

Reviewer #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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Rebuttal for_COMMS-BIO-26-0880

Reviewer #1

The data are interesting and confirm the previously shown contribution of Kv7 channels to the pathophysiology of FXS. The results are generated using sufficient number of animals and although the findings are often marginal with high variation, they are well described. The discussion remains limited related to the potential mechanisms underlying differences observed between mouse and rat. The manuscript is carefully written and the text is fluent (only in Results one misspelling appeared as "in" twice). Based on the previous studies and the interesting new AIS results, the differences obtained may have impact and particularly affect the developmental trajectories, considered as the main message of the manuscript.

We thank the reviewer for the overall positive feedback.

The following issues remain to be addressed:

- Since the electrophysiological properties can be region- and cell-type dependent (Brandalise et al., 2020; Kalmbach et al., 2015) and Kv7 channels are widely expressed in neurons, it would be important to determine Kv7 expression in the rat FXS model as done in *Fmr1* KO mice.

To address this concern, we have performed staining against Kv7.2 in brain slices from P12-15 WT vs Fmr1 KO rats and assessed hippocampal sub-regional expression patterns (new Fig. S6). These new results reveal that 1) within the rat, Kv7.2 is expressed throughout the entire hippocampus and 2) that there are no obvious differences in expression patterns between WT and Fmr1 KO.

We agree that testing cell population differences in Kv7.2 expression would be an interesting experiment. However, as our recordings were carried out exclusively in pyramidal neurons, the primary neuronal sub-population within CA1, we believe this to be outside the scope of our current study.

- In results text needs to be revised to study "conserved abnormalities P6-10 week".

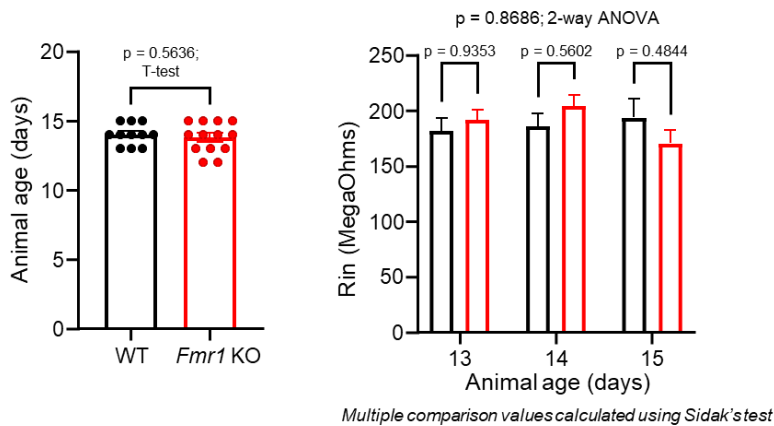
We apologise, but we are unsure what the reviewer means by this comment. Since this is a request for textual alterations, we hope that this could be clarified during review of the revised manuscript?

- Is the high variation in results at P12-15 caused by developmental change and could more accurately determining the day improve results? Another developmental time point for the studies would be needed.

It is true that day-to-day developmental changes can cause variability, particularly in our P12-15 cohort, where neurodevelopment is so rapid that even differences of a single day can potentially result in differences in neuronal properties. While this may explain some of the variability in our data, we would like to clarify that the age distribution of both genotype groups was very similar, and thus any potential effects of age have been balanced (see Figure on left below).

As an additional validation, we have plotted the input resistance, a key readout of neuronal excitability, against corresponding animal age and present this data in the

below figure on the right for the reviewer. We have left out datapoints for P12 animals as the only P12 animals in this cohort were two KOs and thus there is no WT data. However, note that input resistance is stable across age and genotype, and thus we do not expect variations in age to be a major contributor to our data.



Legend: left- comparison of the postnatal age (in days) of rats from our P12-15 cohort split by genotype. Right- comparison of input resistance (a measure of excitability) group by postnatal age across both genotypes. Note that data for P12 animals was omitted here as no WT data are available. Data are mean $\pm$ SEM.

While we agree that another developmental timepoint would be interesting to study, this would be extremely time consuming to complete and we are not convinced that it would add major new findings to our manuscript. For example, if we were to repeat experiments at P21, we would likely see that the phenotypes observed at P12-15 in the knockouts are still present but less prominent, in line with our observation that there is a developmental delay in the Fmr1 KO. Thus, while we are thankful for this suggestion, we feel that the time required does not match the benefit of performing this additional work.

- The differences between mouse and rat have to be better explored.

Similar to our rebuttal above, we agree that this would be interesting, but we feel this is an unrealistic request given the current scope of the manuscript. For example, repeating the whole study in another species would take several years. To address this point we have however, expanded and refocussed our discussion so that there is greater coverage of species differences in Fmr1 KO rodents (P26; paragraphs 2 and 3).

- In figure legend N/n values are given and although it is indicated in Materials and methods, it would be good to indicate it at least in the first figure legend, too.

We agree this would be helpful to all readers and have thus specified what n/N refers to in each figure legend (e.g. neurons / rat).

Reviewer #2 (Remarks to the Author):

In this study, Webster et al examine a potential role for Kv7 channels in excitability alterations in a rat model of Fragile X Syndrome, the *Fmr1* knockout (KO). Importantly, they find changes in juvenile animals at P12-15 that have recovered by adult stages. Careful electrophysiological experiments define changes to repetitive action potential (AP) firing, with decreased firing rates at high levels of current injection paralleled by progressive widening of AP width (relative to the start of injection). In addition, they find altered handling of synaptic vesicle pools, with a faster replenishment rate in the *Fmr1* KO neurons. To various extents, these alterations seem to be at least partially mediated by dysfunction in Kv7 potassium channels. Overall, this is a very well carried out study which adds to the growing literature on excitability changes and indeed the part played by potassium channels in models of Fragile X syndrome. I have several minor issues which could be addressed by the authors.

We are very pleased to hear that the reviewer appreciates our work and are thankful for their positive feedback.

Some of the results are quite confusing, which is rightly acknowledged by the authors in the discussion. The data seem to point to dysfunction of Kv7 channels, but this only seems to be reflected in a wider AP (not a usual feature of Kv7 loss of function) without any of the expected effects of decreased Kv7 function, such as a more depolarized resting membrane potential, decreased AP threshold etc, and indeed one would predict increased firing rates, but in fact they find the opposite – that firing rates are decreased (albeit at high current injections). Even more strangely, while Kv7 blockade has the predicted effects on both genotypes, indicating that Kv7 channels are present and active, Kv7 activation has no effect on the *Fmr1* KO neurons. I am not sure I am convinced by the explanation provided that perhaps there is functional decoupling – if this were the case, you might expect that blockade would have no effects.

We appreciate the reviewer's comments and accept that there may be other factors at play underlying the phenotypes we observe beyond full functional decoupling of Kv7 channels. Nonetheless, we believe the additional data that we have now included in our revised manuscript add weight to this hypothesis. Primarily our new M-current data, which reveals that Kv7 channels appear entirely functionally intact in our model (new Fig. 7; described in text P24; lines 697-708). Therefore, some form of functional decoupling of these channels appears the most likely mechanism. We have updated our discussion to cover this in greater detail.

While I believe the data 'is what it is' and I suspect there may be something more complex and interesting underlying this, a key experiment that would be worth checking is to measure the M current itself (WT vs KO, as well as after Kv7 agonism/antagonism). This would also be a much better way to assess what has happened to Kv7 function versus immunostaining which is notoriously difficult to quantify and see differences with.

As discussed above, these experiments have now been completed and reveal that Kv7 channels are functionally intact.

It would also be very helpful if the authors could include the data showing the effects or not of Kv7 activation / blockade on RMP and AP threshold. Finally, please add baseline, actual, single AP width data – while this is not expected to be changed, it is important given the later results.

These parameters have now been added to our manuscript (new Tables S3-S6). The rheobase single AP half-width data is also included in our manuscript (new Table S1) and is indeed unchanged. This has been clarified within the text (P11; lines 353-354).

The authors examine AIS morphology in dissociated cultures, citing ‘similar.. defects’ from their previous work. However, this does not examine excitability at all, and there can be big differences between dissociated culture and more intact preparations at this level. I would suggest that it should be straightforward to analyse AIS morphology at least in sections of the hippocampus, which would add much weight to these conclusions, and it would be worth trying to also image Kv7.2, although I appreciate this might be more difficult.

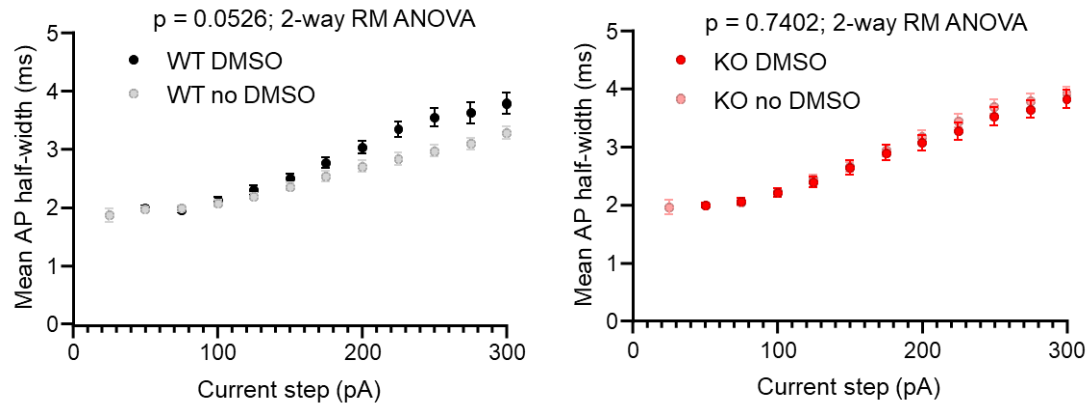
The reviewer makes a very valid argument and we thank them for this suggestion. We have now performed staining against Ankyrin-G in fixed slices from P12-15 rats. While we do not observe obvious differences in AIS morphology, there is a reduction in the density of labelled AIS, which taken together with the neuronal culture data in our original draft, points to altered development of the AIS in Fmr1 KO rats (new Fig. 6). We have updated the abstract, results (P21; 2nd and 3rd paragraphs) and discussion (P26; last paragraph & P27; lines 777-781) of the manuscript to reflect this. As the reviewer acknowledged, Kv7.2 staining of the AIS was not reliable in slices due to poor penetration of the antibody and thus we have excluded this data.

Note that previous work from our institute (Asiminas et al., Molecular Autism, 2022. doi: 10.1186/s13229-022-00528-z.) has already examined CA1 AIS length in 2–3-month-old Fmr1 KO rats and observed no difference, in line with what we would predict from our core observation that CA1 phenotypical defects are lost in adolescent rats. Therefore, we have not performed these experiments in 6-10 week old rats but have highlighted this within the discussion (P26; lines 751-754).

For the experiments with the Kv7 agonists/antagonists, although the WT and KO firing rate and AP width graphs are not overlaid, I wonder if there are still significant changes seen (in the no drug controls)? If not, this might account for some of the surprising findings. It might also be as a result of variability (likely higher in rats) and perhaps these are not littermate experiments, but if so, this could be mentioned.

We acknowledge that indeed the phenotypical defects appear to be lost in the no-drug controls. These animals are age-matched littermates, and thus we do not believe this to be the cause of this discrepancy. Rather, we believe that DMSO itself is occluding the phenotype. Note that previous work has indeed confirmed that DMSO can have substantial effects on neuronal properties even at concentrations lower than the 0.1 % we used (Tamagini et al., PLOS One, 2014. DOI 10.1371/journal.pone.0092557.). We present data in support of this in the below figure for the reviewer. Note the observation that DMSO appears to increase AP half-width only in WT rats such that it is increased to the level of KO animals. We suspect that as the AP half-width in the

KO's is already increased relative to WT's, there is essentially a 'ceiling effect' where it cannot go any higher, hence the lack of DMSO effect on the KO's. Importantly, we would highlight that DMSO was kept constant at 0.1 % for all experiments and thus the effects we observe with K_v7 activation / blockade are true effects of the drug, and not an artefact of DMSO.



Legend: AP half-width across increasing depolarising current steps in WT (left) and KO (right) with and without 0.1 % DMSO in both genotypes. Data are mean ± SEM.

The main text is not in the same order as the figure panels, which makes it somewhat confusing for the reader. I recommend matching them. Personally, I also think it would be easier to discuss first the retigabine experiments and then XE991.

Upon re-reading our manuscript we agree that this is confusing and have thus reordered the text so that it directly follows the figures (P17; paragraphs 2 & 3).

Appropriate sample sizes are used throughout, but please make it clear in the first figure legend what the notation means – I assume it is cells/animals, but this should be added.

We have implemented this change to all figure legends, as reviewer 1 has also suggested.

P6, 1st paragraph, line 4. 'In agreement...' – would be good to rephrase so that it is clear that this is referring to previous work. Eg 'previous work in mice has shown....'

P7, para 4. 'The rationale being that roscovitine...' - this is not a sentence.

P13, line 1 – no apostrophe after its.

All of these changes have been implemented.

Reviewer #3 (Remarks to the Author):

The authors set out to characterize intrinsic excitability of CA1 pyramidal neurons and presynaptic properties in a *Fmr1* KO rat model of Fragile X syndrome with a particular focus on two developmental time windows. Key findings include an action potential broadening and enhanced SV pool replenishment observed in P12-15 *Fmr1* KO rats which both do not persist into adolescence. Furthermore, they propose a potential link to altered Kv7 channel function based on axon initial segment remodelling and channel redistribution.

The paper is very interesting, however several points should be addressed and clarified, please.

We are thankful to the reviewer for the positive feedback and encouraging remarks.

Were the recordings performed in dorsal or ventral hippocampus?

They were performed in the dorsal hippocampus. This has been clarified in our manuscript (methods: P4; lines 95-96 and results P11; line 342).

The terminology “loss of Kv 7 function” is confusing to me since usually reduced Kv7 function is associated with increased excitability and not reduced excitability as presented here.

This comment is addressed in detail below.

Fig. 1:

Can you please speculate why rheobase and input resistance are unchanged despite the strong trend to hypoexcitability of KO CA1 pyramidal neurons?

Note that there is a ‘weak trend’ towards an increase in input resistance in the knockout ($p = 0.1218$). We appreciate this may appear at first counterintuitive. However, we believe the discrepancy here relates to the interpretation of hypoexcitability as we will outline below.

Fig. 2, Fig. 4 and Fig. 5:

The authors link hypoexcitability shown in Fig. 2 to an altered function of Kv7 channels. Hypoexcitability would be compatible with an increased Kv7 function. Therefore, in my mind the next logical experiment would have been a rescue attempt with XE991 to check whether the hypoexcitability can be reverted. Whereas the authors interpret the data shown in Fig. 5 A and B as an indication of enhanced excitability due to left-shifted input-output curves in presence of XE991, I actually rather had the impression that the number of fired APs further declined in presence of XE991 for current injections above 250 pA and remained basically unchanged or only marginally increased for smaller injections. Also, according to the statistical tests data only showed a trend. An important point might be that hypoexcitability for the KO in comparison to the wildtype emerges only for current injections of 300 pA and more, when neurons increasingly start to plateau. XE991

seems to amplify this effect due to Kv7 blockage, associated reduction of M-current and reduced spike-frequency-adaptation in combination with electrophysiological immaturity (in contrast to the juvenile time point at which current injections up to 500 pA are tolerated without AP breakdown/plateauing). Can you please clarify this.

We thank the reviewer for raising this issue. We believe that all of the above can be explained because the neurons are not truly hypoexcitable. Note that as mentioned above there is a slight trend towards an increase in input resistance, which if true would indicate hyperexcitability. We believe that the neurons are entering a state of depolarisation block at higher current injections, where they are unable to accommodate action potential firing (most likely due to a combination of sustained inactivation of Na channels and K channel dysfunction). This may be interpreted as hypoexcitable in the sense that they fire less action potentials, but our data suggest they are that they are not truly 'less excitable'. Indeed, if this were true, one might expect that the KO neurons would fire less action potentials along the whole input-output curve, and not only after reaching plateau. We believe this interpretation would be far more consistent with existing literature on FXS, where CA1 is known to be hyperexcitable. We have not altered our manuscript to include this discussion due to word count limitations.

Expectedly, retigabine decreased the AP firing in the wildtype condition (Fig. 4B) but it also affected AP firing in the KO. This effect occurred primarily for current injections below 300 pA and was absent for higher current injections (thereby all in all displaying a different pattern than the one with XE991 and also a different pattern than the one seen when comparing KO and wildtype). I agree that the retigabine effect is less pronounced in KO neurons. Yet, XE991 appears to cause comparable effects in wildtype and in KO. Taken together, I feel that additional features such as altered mAHP and spike-frequency-adaptation in KO versus wildtype could help to substantiate the role of Kv7. Did you attempt to analyze these parameters?

These parameters are included in our revised manuscript (new Tables S3-S6). Note that barring apparent minor genotype-specific effects of XE991 on RMP and fAHP, these parameters are unaltered. Retigabine did not influence these parameters in any observable way (new Tables S3-S4).

On the other side, M-current recordings (could be estimated by recordings before and after XE991) would provide a more direct confirmation of a role of Kv7 channels. In case M-current was indeed reduced at P12-15 it would be interesting to see whether M-current normalizes in the juvenile time window.

We have recorded the M current in P12-15 rats and include this data in our revised manuscript (new Fig 7; described in text P24; lines 697-708). Note that this appears entirely intact in our model. We believe this adds solid evidence to our hypothesis that the channels themselves are not altered, but rather they are functionally decoupled due to alterations in the AIS where they are strongly expressed.

Furthermore, whereas XE991 broadened the APs in wildtype and KO neurons, retigabine did not rescue the AP half width of KO neurons. I value the effort the authors took to investigate the distribution of Kv7 channels at axons and specifically at the AIS. However, given that a role of BK channels was previously demonstrated in AP broadening (in CA3 of Fmr1 KO mice) I find it hard – based on the presented data, particularly in absence of any BK channel testing and M-current recordings – to conclude that in the rat model reduced Kv7 function represents a major correlate. I feel the study would benefit from an experiment using BK channel modulators to demonstrate that BK channels are actually not the main contributors in the rat model.

We have performed some pilot experiments in a very small sample of WT vs Fmr1 KO slices with the BK channel activator maxipost (also known as BMS-204352) and were surprised to observe that it has no obvious effect of BK activation in either (data not shown). In further support, we analysed the rheobase fAHP in WT vs Fmr1 KO rats as per the reviewers below request, as fAHP is tied to BK channel function, and observed that it was not significantly different between conditions (data shown below and in Tables S1 & S2). Thus, we consider it likely that BK channels are less of a contributor to dysfunction in the rat model than the mouse. This is an important observation and has been expanded upon within our discussion (P26; lines 775-766).

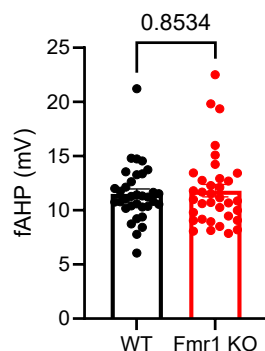


Figure: The rheobase fAHP in WT vs Fmr1 KO rat neurons. Statistical test used was Mann-Whitney.

Was the fAHP actually comparable between wildtype and KO?

Yes it was (see above). This has been added to all intrinsic property tables (Tables S1-S6).

Table S1 shows that the AP half-width is unchanged between wildtype and KO. Were these values derived from the first AP or from the 5th one? Which current step was used for the analysis?

This was the rheobase (ie. first) AP. This has been clarified within the results (P11; lines 353-354).

Given the importance of the retigabine experiments, I wonder whether the authors initially tested different concentrations? 2.5 μ M may have been a bit on the low side.

We did not. This concentration was chosen as a recent paper examining Kv7 dysfunction in Fmr1 KO mice (Luque et al., J. Physiology, 2024. doi: 10.1113/JP285244.) observed that 2.5 μ M was the concentration that permitted observable differences between WT and KO neurons without eliciting a ‘ceiling effect’. Furthermore, we also point to the new M-current experiments we have included, which indicate robust Kv7 activation with 2.5 μ M retigabine. Thus, this concentration appears very effective.

Did I get this right that retigabine is thought to also have a direct effect in the presynaptic compartment/on presynaptic glutamatergic function, dissociated from its effect on AP firing (soma/AIS)? The synaptic effect seems to rather align with an increase of excitability, which would be an undesired effect for an antiseizure drug. Can you please comment on this, since this may have broader implications.

We thank the reviewer for this very insightful comment. Upon reviewing the literature, it does appear that there is substantial evidence that Kv7 channels are expressed presynaptically. However, these papers suggest that presynaptic Kv7 expression constrains neurotransmission rather than facilitates it. We thus believe this to be an unlikely explanation for our data.

Aside from the axon/AIS staining Fig. 6 also shows strong somatodendritic Kv7 signal. Were there any obvious differences between KO and wildtype?

We have performed analysis within both the dendrites (new Fig. S5) and soma (new Fig. S6). There are no changes in somatic expression. There is an apparent reduction in expression specifically within the dendrites of cultured neurons. We could not recapitulate this in intact circuits (fixed slices) and therefore consider it unlikely that this explains the pronounced loss of effect of Kv7 activation in the KO.

In Fig 4Bi (upper left panel, DMSO wildtype) the box around the APs appears to be misplaced.

We apologise but we could not identify this error. Is the reviewer suggesting that the inset (Fig. 4Ci) does not match the placement of the box (Fig 4Bi)? If clarified, we can adjust this.

Rebuttal for COMMS-BIO-26-0880

Please refer to the tracked version of the revised manuscript when viewing highlighted changes.

Reviewer #1

The revised manuscript includes many new data and the difference between mouse and rat data is better discussed. However, the mechanisms underlining the species-specific difference in the results remain open.

We thank the reviewer for raising this point. The most prominent species-specific differences between the Fmr1 KO mouse and rat appear to be:

- 1) The mechanism of K_v7 channel function: for which we observe a pronounced loss-of-effect of K_v7 channels in rat; whereas in mouse these channels appear to be functionally intact to the point that K_v7 activation can rescue circuit hyperexcitability (Luque et al., J. Physiol, 2024).***
- 2) AIS length: which appears to be longer in the Fmr1 KO mouse relative to WT (Booker et al., Cell Rep., 2020) but shorter, or perhaps developmentally delayed, in the rat.***

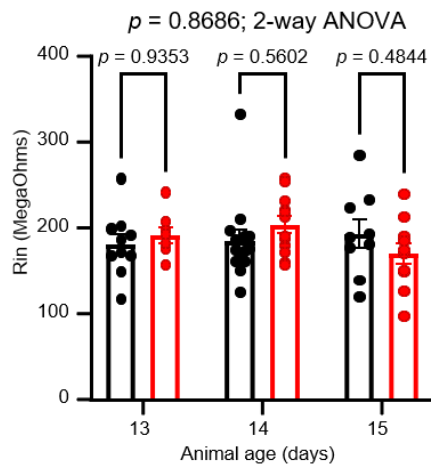
We believe that these two discrepancies are likely linked: if our hypothesis that K_v7 dysfunction is secondary to AIS dysfunction is correct, it would indeed make sense that K_v7 function was more pronounced in the mouse where the AIS is longer, and reduced in the rat where it appears to be immature (our data). This putative mechanistic explanation was covered in the discussion (lines 356-372).

We are not aware of any other species-specific differences. Indeed, the action potential broadening phenotype we observe is consistent with the mouse model (Deng et al., Neuron, 2013), and while we accept that our action potential discharge data (Fig. 2) may at first appear contradictory to the concept of hyperexcitability in Fmr1 KO rodents, we have included a more comprehensive explanation for this apparent discrepancy in the revised manuscript in line with reviewer 2's comment below (lines 373-381). We hope the reviewer considers this clarification sufficient to resolve their query.

The developmental stage-dependent changes are not fully sufficiently justified; input resistance shown in the figure provides actually some evidence of reduced KO values at P15.

We thank the reviewer for raising this issue. We appreciate that the previous reviewer figure could be interpreted as a putative reduction in the mean input resistance between WT and Fmr1 KO rats specifically at P15. For clarity, we have reattached this figure below with each individual data point shown. We would draw attention to the fact that the variability of the datapoints appears very similar between conditions. We would also note that the post-hoc multiple comparison test for P15 returns a p value of 0.4844; far greater than a statistically significant difference in input resistance between genotypes. Finally, we would note that a reduction in input resistance would go strongly disagree with the literature, where neurons are generally thought to be

hyperexcitable in *Fmr1* KO rodents. Therefore, an increase would be expected if there were truly a genotype effect. Thus, while we agree that the reviewer raises an important point, we believe that this putative reduction in input resistance can be explained by random variability in the data rather than a true genotype effect.



Legend: Comparison of input resistance (a measure of excitability) group by postnatal age across both genotypes. Note that data for P12 animals was omitted since no WT data were available. Post-hoc comparison p values are as reported by Sidaks test. Data are mean $\pm$ SEM.

Before publication careful proofreading would be needed i.e. line 80, removed the repeated "in."

Done.

Abbreviations in Methods should be consistent (SI); minutes (line 98, 141, 197, 295), seconds (line 161), hours (line 214, 219, 306).

Thank you for highlighting these inconsistencies. We have now implemented the suggested changes to ensure consistency in our nomenclature.

20% line 122

Line 171 , destruction of the brain?

Line using "free-floating method" for clarity.

Consistent use % without space

Line 252, not recommended to start a sentence with numbers.

All changes implemented.

Reviewer #2

The authors have conducted several new experiments and have addressed all my concerns satisfactorily.

We are pleased to hear that the reviewer appreciates the revised version of our manuscript.

Abstract and line 55 – should spell out that ‘P12’ means postnatal day 12.

Done in both cases.

The explanation given to reviewer 3 about neurons not being hypoexcitable but rather entering depolarization block is helpful, and if there is a way to fit it into the discussion I think will be very helpful for readers, as the data remain a little confusing and surprising for people who usually think of Kv7 loss leading to hyperexcitability.

We have now included this information (lines 492-500).

Reviewer #3

Thank you to the authors for thoroughly addressing my comments.

The authors took great effort by adding entirely new data and considerably revising their manuscript. Hypotheses and conclusions were markedly strengthened. [...] I have no further questions.

We are very grateful to the reviewer for their positive feedback on our revised manuscript, and are pleased that they consider our revisions sufficient to address their initial comments.

Page 17, line 512: “was unchanged in Fmr1 KO synapses” -> “synapses” probably needs to be deleted

This has been changed to ‘neurons’.

Page 22, line 665: “total gross Kv7.2 expression in unaltered” -> is unaltered

Done.

Reviewer #5

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Communications Biology initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

No rebuttal required.