

Huntingtin phosphorylation governs BDNF homeostasis and improves the phenotype of Mecp2 knockout mice

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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

19 June 2019

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript.

Overall, the referees find the study novel and of interest. While ref. 1 is mainly concerned about the clinical relevance of the approach, this referee and the other two referees ask for additional controls, details, and experiments to improve the conclusiveness of the findings and we would like to insist on those. As a proof of concept study, we would be happy to invite revision on the condition that experiments, and missing data be provided and add to the findings. We also would encourage you to improve the *in vivo* data performance and analysis along the ref.1's comments. We do however, realise that this could potentially lead to a lot of additional work and time and as such, should you already have additional *in vivo* data, we would like to see it but won't insist on it as a prerequisite for resubmission.

We would therefore welcome the submission of a revised version within three months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity. Please note that EMBO Molecular Medicine strongly supports a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published, we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

Rett syndrome (RTT) is the first cause of severe intellectual disability in girls and it is generally caused by mutations in MECP2. Although studies on mouse models of this disorder prove that RTT is reversible, at least in mice, no effective cure is yet available. Mecp2 deficiency leads to the deregulation of several genes; among these the down regulation of BDNF is generally accepted as having a role in the disorder. Accordingly, several pre-clinical and clinical trials aim at fostering BDNF expression in RTT. This knowledge, together with a previous publication from the same group demonstrating that HTT modulates BDNF transport, represent the rationale of this novel study. In this manuscript, the authors use genetic and pharmacological approaches to activate HTT, through its selective phosphorylation at S421, thus demonstrating evident amelioration of the Mecp2 null mouse condition.

In general, the technical quality of this work is appropriate, and the whole manuscript is well-written, easy to read and follow and certainly clear also for non-specialists. Conclusions are well supported from data. Although the involvement of BDNF and its modulator HTT are not novel, the presented data and their therapeutic effects on RTT mice are novel. Concerning the medical impact, I have some concerns. Indeed, I believe that too much emphasis has been given so far to BDNF both by the authors in the introduction and generally by the community. As the authors point out, genetic experiments proved that by increasing BDNF levels only some symptoms are ameliorated in mice. In possible good accordance, although clinical trials are still ongoing, first data might indicate only mild improvements. Eventually, in this manuscript the authors use a drug that is actually an aggressive immunosuppressant. Presented data certainly represent a proof of principle of the relevance of S421 in HTT; However, I am not convinced that this molecule might be used in RTT girls.

Eventually, I believe that this work will benefit from some amendments.

In particular:

1. In the abstract the authors suggest the importance of identifying cellular pathways selectively enhancing BDNF signaling in appropriate neuronal circuits. However, in the manuscript they have only analyzed corticostriatal connections. I suggest revising the abstract.
2. Introduction - a) the disorder does not appear only in the second year; b) correct duplications or triplications with duplication or triplication; c) the references Martinowich et al. and Chen et al. 2003 do not prove the involvement of BDNF in the appearance and progression of the disorder; d) I suggest making more clear that rescuing BDNF could not be the definitive solution for RTT.
3. Materials and methods. Although the animal studies make use of quantitative data, in the RTT field is generally recommended to perform blinded studies. However, since the authors do not consider this a pre-clinical study, I believe it is acceptable. What I like much less is the use of homozygous animals for HTT meaning that they never compare siblings. I understand that at this stage there is no solution and that the SA control helps in being confident on the obtained results. The journal should decide whether these approaches are acceptable.
4. All western blots lack MW. Please indicate what are the two PSD95 bands.
5. Supplementary Fig. 3 is pretty bad as it is the case for Fig. 4A. Further, I do not find appropriate to use in the same analysis a different internal standard and run different gels.
6. Fig. 2A is missing all the other genotypes, or at least the WT.
7. Why the authors show the HTT phosphorylation after 2 hrs from FK506 administration while the treatment lasts much longer, and the molecule is administered every 48 hrs? How is HTT phosphorylation when the authors analyze the mouse behavior or at least at the end point of the study?
8. The authors claim that the animals at P30 are symptomatic, but they did not perform any behavioral analysis before treatment (base line of the treated animals); they should at least comment on the gravity of symptoms at this stage.
9. It is not clear to me in the mouse mating procedure, where is the wt male breeder coming from.

Referee #2 (Comments on Novelty/Model System for Author):

Combination of cutting edge in vitro co-culture system in micro fluidic chambers and in vivo Tg mice model.

Referee #2 (Remarks for Author):

In this manuscript the authors demonstrate that promoting HTT phosphorylation at Ser421, restore endogenous BDNF axonal transport in vitro, increased striatal BDNF availability and synaptic connectivity in vivo and improved the phenotype and survival of Mecp2 KO mice, a Rett syndrome model.

This impressive work strengthens and provide new mechanistic insights to previous studies of the same group which demonstrated HTT downregulation in the Mecp2 mice that can be rescued by BDNF.

Overall the data here is convincing, well controlled, the story is in line with their previous results, fit to the aims of EMBO molecular Medicine, and can open new approach for RTT treatments.

Comments:

- 1) It will be interesting to know whether Mecp2-deficient mice exhibit lower HTT phosphorylation compare to WT. Also showing HTT phosphorylation state in human patient samples, will be very relevant.
- 2) It will be beneficial and stronger to show some histology for cell death/survival/synapse in addition to the behavioral tests of the treated mice.

Minor Comments:

- 1) Figure 1: Please add validation for Phospho/non phospho HTT (WB/seq)
- 2) Figure 2: Add WT/KO WT/KO HTTsd/KO HTTsa in all figures
- 3) Figure 3: add the WB control for FK506 proper function (from figure 4 to here).
- 4) All supplementary figures except sup figure 1 are missing titles.

Referee #3 (Comments on Novelty/Model System for Author):

Different experimental replicates have been carried out and the numbers and statistically significant included in the figure legends. Some suggestions for some improvement are included in the comments below.

The authors use both neuronal cultures and a mouse model depleted for the protein which constitutes the highest association to Rett syndrome to date.

This high translational study provides proof that a chemical treatment (easy delivery) has potential therapeutic application for Rett syndrome and other disorders caused by BDNF bioavailability deficits.

Referee #3 (Remarks for Author):

The manuscript "Huntington phosphorylation governs homeostasis and improves the phenotype of Mecp2 knockout mice", by Ehinger et al. proposes a novel strategy to correct or improve disease symptoms in Rett syndrome by the phosphorylation of the Huntingtin protein at the ser 421 in Mecp2 knockout cells and mice, a model for Rett syndrome. The authors show that a Mecp2 knockdown of around 60% causes drastic deficiencies in the transport of BDNF in neuronal cell cultures by reducing both the speed and flow of BDNF vesicles along the axon. BDNF transport defects are prevented by constitutive phosphorylation of HTT at the residue 421 (HTTSD) phosphorylation in vitro and in vivo.

The double mutant mice (Mecp2 ko/ HTTSD) display improved BDNF transport, comparable to that observed in WT mice, as well as increased body weight, increased life span within a given period, reduced apnea, improved motor function and increased night activity. By testing phosphor-TrkB and PSD95 levels the authors report that BDNF restored transport translates in a functional restoration of both BDNF pathway and corticostriatal synapse homeostasis,

The chemical treatment with FK506 (which inhibits calcineurin, responsible to inhibit HTT phosphorylation) had the same BDNF restored transport in culture cells, and phenotype

improvement in the double transgenic mice. The treatment didn't induce any detrimental effect in control mice.

The authors present an interesting and well conducted study supporting that HTT phosphorylation at residue 421 increases the bioavailability of BDNF at the synapse and therefore constitute a directed, rational strategy to consider disorders, like Rett syndrome, where BDNF bioavailability is impaired.

To strengthen the reached conclusions reached and facilitate the reader's understanding, some additional information and modifications should be provided.

Major comments:

- Figure 1. The authors show that HTT phosphorylation at S421 (HTTSD) rescues the transport of BDNF vesicles in siMecp2 neurons. Given the potential interest in therapeutic applications of this strategy, a control of cell viability and homeostasis in this experimental condition is needed.
- Better, cleaner Western blot examples of phospho-TR3KB should be provided in Suppl. Fig. 3. Total TR3KB levels should also be shown (and text modified if necessary).
- Survival curve in Fig. 2B show a time-limited increase in cell survival through a specific window. At the end of the experiment, after day ~90. The authors should provide phosphorylation status of HTT at that later phase of the experiment, or any other evidence that might explain the transient nature of the increased survival effect. The text should modify accordingly to reflect this non-lasting effect.
- Fig 4 support. Show Mecp2 levels in Mecp2 ko mice treated with FK506.
- Are electrophysiological deficits restored in Mecp2 mice after FK506 treatment?

Other comments:

- HTT levels in Suppl. Fig. 1. The Western blot results should be quantified, SD or SEM values calculated, and the number of animals and trials indicated in the figure legend. The text should be updated to reflect the quantification results if necessary.
- Fig. 3 (and text) should reflect the time (after drug treatment) that the shown results are reached. Levels of HTT and HTT phosphor Ser421 in treated cells need to be provided.

Minor comments:

- Page 10: correction of the statement, "Cortical neurons were transduced with mCherry-tagged BDNF lentivirus (BDNF-mCherry) and we used using spinning disk confocal videomicroscopy to record the dynamics of BDNF-mCherry containing vesicles within microchannels in the axons (Fig. 1A and Supplement Movie 1)".
- Page 10: Supplementary Fig. 1A is mislabeled as Suppl Fig. 2A in the figures section. Same applies for Suppl. Fig 1B (in text) and Suppl. Fig 2B (in figures section). Please label figures correctly for consistency and the easier read of the paper (first figures to mention first).
- Page 11. Supplementary Fig. 1 should be labeled as "2" since it appears in the manuscript second.
- Page 13. Supplementary Fig 2G should be labeled as 4G.

1st Revision - authors' response

18 October 2019

Reviewer 1

Rett syndrome (RTT) is the first cause of severe intellectual disability in girls and it is generally caused by mutations in MECP2. Although studies on mouse models of this disorder prove that RTT is reversible, at least in mice, no effective cure is yet available. Mecp2 deficiency leads to the deregulation of several genes; among these the down regulation of BDNF is generally accepted as having a role in the disorder. Accordingly, several pre-clinical and clinical trials aim at fostering

BDNF expression in RTT. This knowledge, together with a previous publication from the same group demonstrating that HTT modulates BDNF transport, represent the rationale of this novel study. In this manuscript, the authors use genetic and pharmacological approaches to activate HTT, through its selective phosphorylation at S421, thus demonstrating evident amelioration of the Mecp2 null mouse condition.

In general, the technical quality of this work is appropriate, and the whole manuscript is well-written, easy to read and follow and certainly clear also for non-specialists. Conclusions are well supported from data. Although the involvement of BDNF and its modulator HTT are not novel, the presented data and their therapeutic effects on RTT mice are novel.

Concerning the medical impact, I have some concerns. Indeed, I believe that too much emphasis has been given so far to BDNF both by the authors in the introduction and generally by the community. As the authors point out, genetic experiments proved that by increasing BDNF levels only some symptoms are ameliorated in mice. In possible good accordance, although clinical trials are still ongoing, first data might indicate only mild improvements. Eventually, in this manuscript the authors use a drug that is actually an aggressive immunosuppressant. Presented data certainly represent a proof of principle of the relevance of S421 in HTT; However, I am not convinced that this molecule might be used in RTT girls.

We thank the reviewer for their insightful summary, their appreciation of the novelty of the study, and their open-mindedness toward the work given their very reasonable concerns about its medical impact. We view this as a proof-of-concept study and hope it lays an important foundation for future work.

Eventually, I believe that this work will benefit from some amendments.

1. In the abstract the authors suggest the importance of identifying cellular pathways selectively enhancing BDNF signaling in appropriate neuronal circuits. However, in the manuscript they have only analyzed corticostriatal connections. I suggest revising the abstract.

We have modified the Abstract accordingly.

2. Introduction - a) the disorder does not appear only in the second year; b) correct duplications or triplications with duplication or triplication; c) the references Martinowich et al. and Chen et al. 2003 do not prove the involvement of BDNF in the appearance and progression of the disorder; d) I suggest making more clear that rescuing BDNF could not be the definitive solution for RTT.

We have modified the introduction as suggested.

3. Materials and methods. Although the animal studies make use of quantitative data, in the RTT field is generally recommended to perform blinded studies. However, since the authors do not consider this a pre-clinical study, I believe it is acceptable. What I like much less is the use of homozygous animals for HTT meaning that they never compare siblings. I understand that at this stage there is no solution and that the SA control helps in being confident on the obtained results. The journal should decide whether these approaches are acceptable.

We agree that littermate controls are essential for any behavioral studies comparing genotypes or treatments. We want to point out, however, the tests concerning the HTT phenotype and FK506 treatment were indeed performed blinded. We have clarified this in the material and methods section, page 6.

4. All western blots lack MW. Please indicate what are the two PSD95 bands.

Molecular weights have been added. In addition to the band at 95 kDa protein, western blotting for PSD-95 identifies a lower band (~70 kDa). This lower molecular weight band appears to be a truncated form of PSD-95 lacking the N terminus, as it has been previously noted in several other studies (Topinka & Bredt, 1998; Schlüter et al, 2006; Xu et al, 2008). In this study, we used an antibody against the C terminal part, therefore revealing the truncated form of PSD-95.

5. Supplementary Fig. 3 is pretty bad as it is the case for Fig.4A. Further, I do not find appropriate to use in the same analysis a different internal standard and run different gels.

We have performed new western blots using the same controls, shown now in a new supplementary Figure 3.

6. *Fig. 2A is missing all the other genotypes, or at least the WT.*

All genotypes are now presented in new Figure 2A and as well as in new supplementary Figure 2. The text was modified to mention these results (see page 13).

7. *Why the authors show the HTT phosphorylation after 2 hrs from FK506 administration while the treatment lasts much longer, and the molecule is administered every 48 hrs? How is HTT phosphorylation when the authors analyze the mouse behavior or at least at the end point of the study?*

We thank the reviewer for raising this point. We analyzed phosphorylation levels after 20 days of injections with FK506. This is exactly the same experimental condition as the one used for behavioral analyses. However, at this time point we did not detect elevated HTT phosphorylation in the samples treated with FK506 relative to DMSO-treated animals, likely because repeated treatments desensitize the pathway. Nevertheless, the key observation remains: chronic treatment of FK506 induces neuroprotection in animals and this effect is lost when HTT cannot be phosphorylated at S421 (see new Figure 4).

We kept the western blot showing positive effects of FK506 after acute treatment in Mecp2 KO mice (new Figure 4A) and have moved the acute treatment of WT and HTT_{SA} mice into a new supplementary Figure 3A. We now provide the effect of FK506 chronic treatment as a new Supplementary Figure 3B.

8. *The authors claim that the animals at P30 are symptomatic, but they did not perform any behavioral analysis before treatment (base line of the treated animals); they should at least comment on the gravity of symptoms at this stage.*

This is a valuable suggestion. We have added the following text to the results section page 16: « We choose P30 as a starting point, since the onset of the Mecp2-knockout phenotype is already apparent at this stage as breathing abnormalities, locomotor deficits and body weight loss (Guy *et al*, 2001; Viemari *et al*, 2005; Matagne *et al*, 2017)».

9. *It is not clear to me in the mouse mating procedure, where is the wt male breeder coming from.*

We modified the sentence in the Material and Methods section page 5 to clarify: “The Mecp2^{tm1-1Bird} Mecp2-deficient mice were obtained from the Jackson Laboratory and maintained on a C57BL/6 background by using C56BL/6J male breeders (also from the Jackson Laboratory).”

Reviewer 2

In this manuscript the authors demonstrate that promoting HTT phosphorylation at Ser421, restore endogenous BDNF axonal transport in vitro, increased striatal BDNF availability and synaptic connectivity in vivo and improved the phenotype and survival of Mecp2 KO mice, a Rett syndrome model.

This impressive work strengthens and provide new mechanistic insights to previous studies of the same group which demonstrated HTT downregulation in the Mecp2 mice that can be rescued by BDNF.

Overall the data here is convincing, well controlled, the story is in line with their previous results, fit to the aims of EMBO molecular Medicine, and can open new approach for RTT treatments.

We thank the reviewer for their appreciation of the work.

1) *It will be interesting to know whether Mecp2-deficient mice exhibit lower HTT phosphorylation compare to WT. Also showing HTT phosphorylation state in human patient samples, will be very relevant.*

We now provide a new supplementary Figure 3B, a western blot of HTT phosphorylation levels in WT and Mecp2 KO mice showing that there is no difference between the two genotypes.

RTT human samples would be highly relevant to this study but, unsurprisingly, are very difficult to obtain. Even so, our experience in using human samples from HD patients has shown that variability of post-mortem preparations makes it extremely difficult to obtain reliable signals for HTT phosphorylation.

2) *It will be beneficial and stronger to show some histology for cell death/survival/synapse in addition to the behavioral tests of the treated mice.*

As suggested by the reviewer, we now provide a full new figure (Supplementary Figure 4) in which we analyzed the presence of cleaved-caspase-3 and TUNEL in Mecp2 KO mice and the effect of FK506 treatment. We did not detect any cell death in Mecp2 KO mice nor any increase in cell death after 20 days of chronic treatment. We now report on page 16: “We next investigated cell death in the striatum of these mice. In accordance with previous reports (Armstrong, 2005; Kishi & Macklis, 2004; Reiss *et al*, 1993; Roux *et al*, 2007), we did not detect any cell death in Mecp2 knockout mice. Importantly, FK506 treatment did not induce any neuronal toxicity or cell death after 20 days of treatment (Supplementary Fig. 4A and B).”

We also investigated cFos as a marker of neuronal activity. We observed a trend toward reduced cFos staining in Mecp2 knockout that was restored to control levels in treated mice. We present these results in the new Supplementary Fig. 4C and D and on page 16.

Minor Comments:

1) *Figure 1: Please add validation for Phospho/non phospho HTT (WB/seq)*

We previously reported that S to A mutation at S421 leads to loss of HTT phosphorylation as revealed by the use of a phospho-S421 antibody (Colin *et al*, 2008). We provide evidence in the new supplementary Figure 1C and supplementary figure 3A that the S421A mutation abrogates recognition of phosphorylated HTT by the phospho-specific HTT antibody. This validation of the phospho/non phospho HTT is provided in the text (page 12).

2) *Figure 2: Add WT/KO WT/KO HTTsd/KO HTTsa in all figures*

We now provide all the different genotypes in new Figure 2 and in new supplementary Figure 1G. The text was modified accordingly (page 13).

3) *Figure 3: add the WB control for FK506 proper function (from figure 4 to here).*

The western blotting analysis of previous Figure 4A showing FK506 effect on WT mice was moved to supplementary Figure 3A as requested by Reviewer 1, as these experiments were performed in mice and not in neuronal cultures.

4) *All supplementary figures except sup figure 1 are missing titles.*

We apologize for this. Titles have been added to all the supplementary figures.

Reviewer 3

The manuscript "Huntington phosphorylation governs homeostasis and improves the phenotype of Mecp2 knockout mice", by Ehinger et al. proposes a novel strategy to correct or improve disease symptoms in Rett syndrome by the phosphorylation of the Huntingtin protein at the ser 421 in Mecp2 knockout cells and mice, a model for Rett syndrome. The authors show that a Mecp2 knockdown of around 60% causes drastic deficiencies in the transport of BDNF in neuronal cell cultures by reducing both the speed and flow of BDNF vesicles along the axon. BDNF transport defects are prevented by constitutive phosphorylation of HTT at the residue 421 (HTTSD) phosphorylation in vitro and in vivo.

The double mutant mice (Mecp2 ko/ HTTSD) display improved BDNF transport, comparable to that observed in WT mice, as well as increased body weight, increased life span within a given period, reduced apnea, improved motor function and increased night activity. By testing phosphor-TrkB and PSD95 levels the authors report that BDNF restored transport translates in a functional restoration of both BDNF pathway and corticostriatal synapse homeostasis,

The chemical treatment with FK506 (which inhibits calcineurin, responsible to inhibit HTT phosphorylation) had the same BDNF restored transport in culture cells, and phenotype

improvement in the double transgenic mice. The treatment didn't induce any detrimental effect in control mice.

The authors present an interesting and well conducted study supporting that HTT phosphorylation at residue 421 increases the bioavailability of BDNF at the synapse and therefore constitute a directed, rational strategy to consider disorders, like Rett syndrome, where BDNF bioavailability is impaired.

We appreciate the reviewer's interest in this work.

To strengthen the conclusions reached and facilitate the reader's understanding, some additional information and modifications should be provided.

Major comments:

- Figure 1. The authors show that HTT phosphorylation at S421 (HTT_{SD}) rescues the transport of BDNF vesicles in siMecp2 neurons. Given the potential interest in therapeutical applications of this strategy, a control of cell viability and homeostasis in this experimental condition is needed.

We agree with the importance of demonstrating the lack of toxicity in our transport assays. Therefore, we performed cell viability assays using MTT. Tests were performed on neurons from different genotypes cultured in the same condition as the transport assays shown in Figure 1. We found that neither HTT genotype (WT, HTT_{SD} or HTT_{SA}) nor Mecp2 silencing had any effect on cell viability. The effects on transport are therefore not due to differences in cell viability. See new Figure 1C and F.

- Better, cleaner Western blot examples of phospho-TrkB should be provided in Suppl. Fig. 3. Total TrkB levels should also be shown (and text modified if necessary).

We have improved the quality of the western blots that are now shown in the new supplementary Figure 1G.

Total TrkB would have been interesting to quantify. Unfortunately, microdissection of the cortex and striatum in our mice (KO, WT, KO/HTT_{SA} and KO/HTT_{SD}) collects less than 100 micrograms of tissue. With the samples obtained we were able to quantify Phospho TrkB, PSD95 and GAPDH, but we no longer have enough to perform the total TrkB assay.

- Survival curve in Fig. 2B show a time-limited increase in cell survival through a specific window. At the end of the experiment, after day ~90. The authors should provide phosphorylation status of HTT at that later phase of the experiment, or any other evidence that might explain the transient nature of the increased survival effect. The text should modify accordingly to reflect this non-lasting effect.

We believe the reviewer is referring to the limited increase in mouse, not cell, survival. The specific window of survival mentioned by the reviewer likely reflects a shift in the survival of the KO HTT_{SD} mice compared to the KO mice. Indeed, although the HTT_{SD} mutation ameliorates behavior and extends the lifespan of the mice, the mutation is only protective until a certain time point. Importantly, the KO HTT_{SD} survival curve never crosses the KO survival curve and the effect is statistically significant. The text has been modified in the results section (page 13). It is important to note that FK506 treatment was not transient and significantly prolonged lifespan (Figure 4B).

The reviewer is also requesting an analysis of HTT phosphorylation status. However, given the nature of the mutation, (S into A or S into D), the phospho-epitope on S421 is absent in these mice (new supplementary Figure 1C).

- Fig 4 support. Show Mecp2 levels in Mecp2 ko mice treated with FK506.

We are not sure we understand the reviewers' concern: Mecp2 protein cannot be detected in Mecp2 KO mice (Guy et al., 2001). If the reviewer is asking for Mecp2 protein levels in WT mice with FK treatment, we provide this information in the new supplementary Figure 3C. Briefly, there are no differences in Mecp2 levels between treatments.

-Are electrophysiological deficits restored in Mecp2 mice after FK506 treatment?

This is an interesting study that we intend to pursue in the future, but we believe that this is beyond the scope of the current manuscript.

Other comments:

- *HTT levels in Suppl. Fig. 1. The Western blot results should be quantified, SD or SEM values calculated, and the number of animals and trials indicated in the figure legend. The text should be updated to reflect the quantification results if necessary.*

The western blot of old supplementary Figure 1 has been performed again and was quantified. It is now shown in the new supplementary Figure 1C and the text (page 12) and legend modified accordingly.

- *Fig. 3 (and text) should reflect the time (after drug treatment) that the shown results are reached. Levels of HTT and HTT phosphor Ser421 in treated cells need to be provided.*

The western blot of the previous supplementary Figure 5 showing levels of HTT and HTT phosphorylation has been moved to the new Figure 3A, with quantification.

Minor comments:

- *Page 10: correction of the statement, "Cortical neurons were transduced with mCherry-tagged BDNF lentivirus (BDNF-mCherry) and we used using spinning disk confocal videomicroscopy to record the dynamics of BDNF-mCherry containing vesicles within microchannels in the axons (Fig. 1A and Supplement Movie 1)".*

- *Page 10: Supplementary Fig. 1A is mislabeled as Suppl Fig. 2A in the figures section. Same applies for Suppl. Fig 1B (in text) and Suppl. Fig 2B (in figures section). Please label figures correctly for consistency and the easier read of the paper (first figures to mention first).*

- *Page 11. Supplementary Fig. 1 should be labeled as "2" since it appears in the manuscript second.*

- *Page 13. Supplementary Fig 2G should be labeled as 4G.*

We thank the reviewer for pointing out these typos, which we have now corrected.

Literature cited

- Armstrong DD (2005) Neuropathology of Rett syndrome. *J. Child Neurol.* **20**: 747–753
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Xu W, Schlüter OM, Steiner P, Czervionke BL, Sabatini B & Malenka RC (2008) Molecular dissociation of the role of PSD-95 in regulating synaptic strength and LTD. *Neuron* **57**: 248–262

2nd Editorial Decision

14 November 2019

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now supportive and I am pleased to inform you that we will be able to accept your manuscript pending minor editorial amendments.

I look forward to receiving a new revised version of your manuscript within 2 weeks.

***** Reviewer's comments *****

Referee #1: Suitable for publication

Referee #2: Suitable for publication

2nd Revision - authors' response

27 November 2019

Authors made the requested changes.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Jean-Christophe ROUX and Frederic Saudou

Journal Submitted to: EMBO MOLECULAR MEDICINE

Manuscript Number: EMM-2019-10889

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 χ^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.

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?	No statistical methods were explicitly used to pre-determine sample sizes in animal studies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	All sample sizes were based on those used in similar previous publications. All animal groups are equal or superior to $n=10$.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animal were excluded from animal studies.
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.	Regarding FK506 treatment, KO mice were randomly assigned in groups.
For animal studies, include a statement about randomization even if no randomization was used.	For animal studies involving treatment, KO mice were randomly assigned.
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.	pHTT WB experiments have been done in parallel into 2 different laboratories (in Grenoble and in Marseille) by different persons.
4.b. For animal studies, include a statement about blinding even if no blinding was done	All behavioral tests were performed by personnel blinded for genotype identity.
5. For every figure, are statistical tests justified as appropriate?	For every figure, appropriate statistical tests were used. Data analysis are detailed in the "Statistical analysis" section of Materials and Methods. N, p-values and statistical tests used are presented in figure legends.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	All analyses were performed using GraphPad Prism. For group comparisons normality distribution of the datasets were tested before performing any statistical test by Shapiro's test. In case of non normal distribution, a non parametric test was performed.

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Is there an estimate of variation within each group of data?	yes by Brown Forsythe's test (equivalent with Levene's test)
Is the variance similar between the groups that are being statistically compared?	when ANOVA is used, Brown Forsythe's test verifies that the variance is homogenous. If not, a non parametric test were applied as Kruskal Wallis 's test

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).	citation, catalog number or clone number are provided in the section Material 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.	NA

* 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.	All mouse lines were on a C57BL/6J genetic background. The Mecp2tm1-1Bird Mecp2-deficient mice were obtained from the Jackson Laboratory and maintained on a C57BL/6 background by using C56BL/6J male breeders from the Jackson Laboratory. The HTT knock-in mice were previously generated by inserting a point mutation in exon 9 (AGC>GAC, Ser>Asp called S421D or AGC>GCC, Ser>Ala called S421A) (Thion et al., 2015). To obtain double mutant mice (Mecp2 KO and S421D or S421A), heterozygous females (Mecp2+/-) were crossed with homozygous S421D or S421A males. Animals were housed under standard conditions of temperature (21 ± 2°C) and humidity (55 ± 5%), with food and water ad libitum in a 12:12h day/night cycle.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Experimental protocols were approved by the ethical committee of the Aix Marseille University and the French M.E.N.E.S.R. minister (Permit Number: 02910.02). The experimental procedures were carried out in keeping with the European guidelines for the care and use of laboratory animals (EU directive 2010/63/EU), the guide for the care and use of the laboratory animals of the French national institute for science and health (INSERM). All experiments were made to minimize animal suffering.
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.	ARRIVE guidelines were consulted and applied

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	NA
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).	NA
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).	NA
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 Biomodols (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.	NA

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.	
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