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Rescue of CAMDI deletion-induced delayed radial migration and psychiatric behaviors by HDAC6 inhibitor

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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 27 April 2016

Thank you for the transfer of your manuscript to EMBO reports. I am sorry for the slight delay in getting back to you; we have only now received the full set of referee reports that is copied below.

As you will see, all referees acknowledge that the findings are potentially interesting and novel. However, they also raise several concerns that would need to be addressed in order to strengthen the study and make it suitable for publication here. Given that many concerns are raised by more than one referee, and several relate to quantifications and statistics, I think that all of them should be addressed. This means that extensive revisions are required, and we can potentially prolong the time for revision beyond the usual 3 months.

We would therefore like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. Given the 7 main figures, I suggest that we publish your manuscript as a full article, for which there are no length limitations. Please change the reference style to the numbered EMBO reports style (in EndNote) and include the entire materials

and methods in the main manuscript file. Supplementary information is now called expanded view (EV) at EMBO press, and we can offer 5 expanded view figures. Please upload them as individual files and include the figure legends at the end of the main manuscript file.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Fukuda et al. followed up a previous study (Fukuda, 2010) to investigate the mechanism of CAMDI controlling radial migration. This study illustrates that a novel CAMDI knockout mouse showed delayed cortical migration, abnormal axonal projection and potential behavioral defect. The study demonstrates that CAMDI interacts with HDAC6 and inhibits the activity of histone deacetylase. Tubastatin A, an HDAC6 inhibitor, can rescue the deficits of radial migration and correct most abnormal behaviors in CAMDI-KO mice.

Although the manuscript is interesting and novel, several key points need to be addressed in order to support their conclusion.

Major Comments

1. The developmental phenotypes of CAMDI-KO mice have not been fully characterized in the paper. As neural progenitor proliferation may impact neuronal fate determination and migration in the later stage, it is unclear if CAMDI-KO affects progenitor proliferation and fate specification. Authors should determine the proliferation of progenitors in the whole brain using BrdU labeling or other proliferation markers, such as Ki67 and phosphor-Histone H3 staining. Particularly, the authors have shown that CAMDI is highly expressed in the intermediate zone (THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 52, pp. 40554 -40561, 2010). Does CAMDI-KO affect intermediate progenitors? In page 7, the first paragraph line 4, the sentence "labeling progenitor neurons in the ventricular zone" is confusing. Do authors refer to progenitors or new-born neurons? The delayed migration of BrdU labeled neurons could be explained by slow proliferation. Since authors have done the BrdU labeling, is the overall proliferation rate changed by CAMDI-KO? In addition, to test if CAMDI plays a role in proliferation and cell fate determination, authors should quantify the total number of Cux1+ cells and measure other layer markers, such as foxp2.

2. For all behavioral tests, the gender will significantly affect the results. The authors should indicate how many male and female mice are used in the method. They should do the analysis separately on male and female mice to determine which gender drives the behavioral difference. Moreover, the authors indicated in the methods "Mice were tested sequentially" in different behavioral tests. However, the figure captions seem to indicate that different numbers of mice were used in different experiments. Were these indeed different groups of mice, or were they the same groups, and

different numbers used from each group? If the latter, why were some mice excluded from some experiments?

3. In the open field test, the CAMDI-KO mice show the hyperactive behaviors with similar traveling time as WT mice. Does the open field test indicate CAMDI-KO mice more anxious? Do they spend less time in the center than WT mice?

4. In the social interaction test, it seems that there is a significant difference between the KO mice interacting with the strange mouse vs the empty cage, which would mean the KO mice still prefer the stranger to the empty cage, indicating that the KO mice show normal social behavior. This is not valid to claim a social defect if KO mice can still distinguish an empty cup versus a stranger mouse. Please run the statistics on this.

5. It is interesting that CAMDI overexpression inhibited the HDAC6 activity and upregulated α-tubulin acetylation. Is CAMDI a general inhibitor of HDAC6 or specific to tubulin? Will this physical interaction block HDAC6 enzyme activity with other substrates? The centrosome abnormality will affect both proliferation and the migration. Many papers have shown that HDAC6 involves in multiple cellular events, including proliferation. Will tubastatin A treatment rescue the migration defect possibly through proliferation?

6. Please explain why not to rescue all behavioral defects, and why only select some behaviors for a rescue. It would make sense to include the social interaction test, as you claim before that deficits in this are part of the autism symptom triad.

Minor Comments
1. Figure 1A seems oversaturated. Perhaps turn down the gain on the confocal?

2. Figure 1E, the WT brain section is clearly more rostral than the corresponding KO section. The author should compare sections at similar brain region.

3. The authors mentioned that delayed cortical migration and aberrant axonal projections detected in CAMDI-KO brains are associated with psychiatric disorders, but did not cite any paper supporting this claim.

4. Page 42, figure 4 title, "Figure 4. HDAC6 inhibition by CAMDI regulates γ-tubulin localization at the basal bodies". It should be α-tubulin.

5. Since DISC1 interacts with CAMDI, will DISC1 modulate CAMDI mediated migration? This should at least be discussed.

Referee #2:

In this study, Fukuda et al produce a CAMDI knockout mouse model to recapitulate neuronal migration phenotypes observed previously in a transient knockdown model. The authors also show that knockout mice exhibit hyperactivity, repetitive behavior, and social interaction deficits. To account for a mechanism to the observed findings, an interaction was described between CAMDI and HDAC6 and that loss of CAMDI resulted in reduced levels of g-tubulin and acetylated tubulin. Given these observation, the authors queried whether activation of HDAC6 might drive neuronal migration and behavioral phenotypes. Interestingly, treatment of mice with an inhibitor to HDAC6 rescued these phenotypes along with increasing protein levels of acetylated tubulin from brain cortical lysate. Over all, this is a interesting piece of work that will move the field forward. However, there are several limitations with the story and the logic is somewhat hazy in several parts of the manuscript.

1. Introduction. The authors make an argument using human genetics to indicate that CAMDI and DISC1 are relevant biological targets. Unfortunately, this is currently highly unlikely. The are several noteworthy publications questioning whether the initial Scottish DISC1 pedigree was analyzed correctly, and more importantly, the validity of DISC1 as a major/minor SZ candidate has not been shown to be true (case in point, the 108 loci paper and several others). With regards to

CAMDI, the papers that the authors cite on DISC1 conditioned GWAS indicate that CAMDI (at a p-value=10E-4) is no different from an event occurring by chance. As such, I would focus on the facts in the introduction, rather than speculation (i.e. DISC1 is a major risk factor for psychiatric disorders - this is not a true statement).

2. Behavioral tests. Over all, I find the approach to be appropriate, but the analyses are concerning. It appears that most comparisons are being performed using t-tests (which is fine for some comparisons) and I'm unable to get a sense of the "true" significance. If the authors are comparing 2 genotypes on the open field (and the other assays), I would like to have more information on the statistical analyses (F-values for interactions and main effects). Also, I'm unsure how long the animals have been placed in the OFT; in figure 2B, the total distance for adults is ~20m and in Figure 7b for juveniles, it is ~7m? Some other variable must be different here. Also, why is there a need to compare juveniles and adults? Is there a progressive behavioral and biochemical deficit? Finally, it appears that the mutants travel more distance in the perimeter (another indicator of anxiety)?

3. Two main points of hazy logic to me: a Y2H experiment was performed, but no information was provided about the details of the library (species of the library, tissue context, what CAMDI probe was utilized for the bait, and importantly, what fragment of HDAC6 was identified from the library). All of this information can be used to potentially bolster your specificity argument in Figure 3. The second point of confusion is the use of HeLa cells to show an effect of CAMDI and HDAC6 on g-tubulin and acetylated tubulin levels. The author indicate that knockout mice are healthy and viable with early migration and behavioral phenotypes. Why, now transition to a cervical cancer cell line? While the authors should be applauded for validating some findings using brain lysate, they should really consider a neuronal cell line at the very least (I noticed some information about SH-SY5Y cells in the methods, but nowhere else) for mechanistic details. In addition, conclusions from all blots and images should be quantified. The reader is presented with representative blots and immunohistochemistry, but has no way of understanding what has been repeated.

4. Finally, the authors show a mild migration phenotype and possibly abnormal projection of neurons. It should be examined whether the mice show some more brain phenotypes relevant to psychiatric disorders, such as reduction of parvalbumin, perineuronal nets, oligodendrocyte markers, increase of the expression of inflammation related molecules, and alterations of neuronal morphology (such as fewer and/or smaller spines, poorer dendrites etc). Also, transcriptome or proteome analyses of some brain regions of the KO mice could be done and the results could be compared to those of the post-mortem brains of human patients.

Minor points.

1. The authors mention southern probes, but don't show data

2. Various typos throughout the manuscript (i.e. Page 9 "social interaction test, the compared...")

3. Mol weights should be indicated on immunoblots

Referee #3:

EMBO Reports manuscript
"Rescue of CAMDI deletion-induced radial migration and psychiatric behaviors by HDAC6 inhibitor."
By Fukuda et al.
The authors' laboratory has previously identified CAMDI as an interactor of DISC1 and myosin II. A knock-down of CAMDI was shown to impair centrosome orientation and radial migration of cortical neurons. Here, the authors characterise a CAMDI knock-out mouse. The authors claim that CAMDI knock-out induces a delay in the cortical migration of Cux1+ neurons born at E14.5, which also mis-project to striatal regions. Further, CAMDI KO mice show behavioural abnormalities including social deficits and repetitive behaviours. To elucidate the molecular function, the authors show that CAMDI interacts with HDAC6, which is involved in tubulin acetylation. In overexpression studies, CAMDI and HDAC6 antagonise each other, which is taken as indication

that CAMDI inhibits HDAC6. In vivo inhibition of HDAC6 rescues several defects of the CAMDI knock-out, including rescuing the mis-localisation of cortical neurons, overcompensates tubulin acetylation, and rescues several but not all behavioural abnormalities.

In conclusion, this manuscript offers characterization of a new mouse model and shows interesting data to suggest that CAMDI has a role in tubulin acetylation. Several key conclusions of the paper, however, are not fully supported by the data, and therefore this study requires revisions in order to be acceptable for publication at EMBO reports. Points that should be addressed by the authors are as follows.

1. In Figure 1A, the authors show a clear mis-positioning of Cux1-positive cells in CAMDI KO mice at postnatal day 2. However, when quantifying the positioning of these cells, the authors perform a Student's t test to compare between the WT and KO distribution. When comparing distributions, Student's t test is not an appropriate test. The authors should perform an appropriate statistical test for this eg. two-way ANOVA. This statistical concern also applies to quantifications in Figure 1D and Figure 6B.

2. In Figure 1C, the authors describe the mis-positioning of EGFP-positive neurons at P21 as 'delayed migration', which implies that the neurons will eventually reach their final destination. However, since this time-point is at the end of brain development (P21), further migration unlikely and this rather suggests mis-localisation of neurons to the lower cortical layers. In order to confirm this, and to check if these neurons also assume the identity of the layer they are in, see the mis-projection of their axons, the authors should use further markers of cortical layering to characterise the fate of these mis-positioned neurons eg Cux1, Ctip2. Additionally, on page 8 first paragraph, the authors state that the mis-localised neurons in layer V had the 'properties of layer II/II neurons'. This appears refers to the fact that they are Cux1+ positive, which is in this reviewers' opinion insufficient evidence for this statement.

3. In Figure 1E and Supplementary Figure 2, the authors show that CAMDI-KO mice present incorrect axon pathfinding, with neurons born at E14.5 projecting to the ipsilateral striatum. However the images shown in these figures appear not to be from the same Bregma level in the brain. To clarify this, the authors should provide images of WT and KO at the same bregma level, and clearly show/quantify the callosal projections in both WT and KO and the presence/absence of striatal projections. They should include information about the number of animals used for this analysis.

4. In the middle of page 7, the authors cite a shRNA knock-down of CAMDI. This appears to refer to their previous paper, which should be cited here; otherwise, it should be indicated that this data is 'not shown'.

5. The authors use the open field test in Figure 2A-B to demonstrate locomotor hyperactivity in CAMDI KO. However more information on this test needs to be shown in order to exclude other conclusions that can be drawn from this test. The open field test can also be used to assess anxiety-like behaviors (thigmotaxis), and since the authors already detect an anxiogenic phenotype in the light-dark test (Figure 2I-J) they should show centre time in the open field to verify whether this phenotype is also detected in the open field (it is mentioned in the methods that time in center was measured, however it is not shown in results. This would help clarify whether their anxiety phenotype is interfering with the interpretation of the open field as a hyperactivity defect only. Additionally, in the result section for this figure (page 9), 'data not shown' is cited that the overall travel time remains unchanged. Since the travelled distance increases, does this mean that the mutant mice run faster? Would that really be hyperactivity, if the activity duration is unchanged?

6. Further to point 5, in Figure 2D in the three-chamber test, the time CAMDI KO mice spent exploring the stranger mouse goes down but this does not result in more time spent interacting with the empty cage. The authors should show the total time spent in each chamber, including the neutral centre chamber, to clarify if the mice are actually showing preference for the neutral chamber over stranger mouse and an empty cage, or just spending time in the chambers of the stranger mouse and empty cage without in fact interacting with them. The authors should also clarify in the methods sections what they define as "interaction time".

7. The cage-hang test is taken as a measure for motor coordination (top of page 9, supplementary figure S3). This test measures the strength of the motor units, not their coordination.

8. In Fig 3E, it should be indicated where the borders between the different fragments lie, to make the experiment easier to understand.

9. In Figure 3D, fragment 3 in the bottom panel seems to migrate faster than the other two fragments. This region of the gel is not shown in the top panel, and the reader cannot judge if this fragment binds CAMDI or not.

10. Figure 4A is cited as indication that CAMDI overexpression increases tubulin acetylation. What is this compared to? There are only two non-transfected cells in the right periphery of the micrograph, and their acetyl-tubulin signal does not look so much weaker than that of the upper transfected cell.

11. The Western blots in Fig. 4B-4D need to be quantified, and the significance of the effects should be evaluated with several biological replicates.

12. Figures 4 and 5 are taken as proof that CAMDI inhibits HDAC6. Instead, it is only shown that they antagonise each other: the data could also be explained if CAMDI attracts acetylation rather than inhibiting deacetylation. The interaction of the two proteins is not a proof of direct inhibition; biological complexes can sometimes bring two antagonising activities together. To show inhibition, it would be necessary to demonstrate that CAMDI has no effect on tubulin acetylation in the absence of HDAC6.

13. In Figure 6A and B, the authors show that after Tubastatin A treatment they could rescue the neuronal mis-positioning of Cux1-positive neurons. To further confirm these results, it is recommended that the authors analyze neuronal migration and axonal pathfinding after Tubastatin A treatment, which will better support their data and the fact that Tubastatin A can also rescue some of the behavioral phenotypes observed in CAMDI-KO mice.

14. In Figure 7D, the authors show that P21 CAMDI-KO mice exhibited decreased grooming time, while in adult mice there is no grooming phenotype (page 9, data not shown). This is intriguing and suggests that some aspects of the repetitive behavior phenotype seen in juvenile CAMDI-KO mice are age-dependent and are ameliorated by adulthood without treatment. The authors should comment on this.

15. The authors should comment in the discussion on why Tubastatin A might be improving hyperactivity, impulsive behavior and environmental adaptation but not social behavior in CAMDI-KO mice.

1st Revision - authors' response 29 July 2016

I am respectfully submitting a revised manuscript (Paper #EMBOR-2016-42416-T) entitled "Rescue of CAMDI deletion-induced delayed radial migration and psychiatric behaviors by HDAC6 inhibitor " by T. Fukuda et al., for your consideration for the publication of *the EMBO Reports*.

In the revised manuscript, we demonstrated that delayed migration by CAMDI KO is not due to alterations in cell proliferation and cell fate determination. Statistical analysis and behavioral tests were properly performed according to reviewer’s comments.

We appreciate for helpful comments of reviewers made on our paper and now believe that we could address almost all concerns and revise the paper accordingly.

We include a cover letter indicating a detailed, point-by-point description of these changes in the following.

I am looking forward to receive your decision.

Referee #1:

Fukuda et al. followed up a previous study (Fukuda, 2010) to investigate the mechanism of CAMDI controlling radial migration. This study illustrates that a novel CAMDI knockout mouse showed delayed cortical migration, abnormal axonal projection and potential behavioral defect. The study demonstrates that CAMDI interacts with HDAC6 and inhibits the activity of histone deacetylase. Tubastatin A, an HDAC6 inhibitor, can rescue the deficits of radial migration and correct most abnormal behaviors in CAMDI-KO mice. Although the manuscript is interesting and novel, several key points need to be addressed in order to support their conclusion.

Major Comments

1. The developmental phenotypes of CAMDI-KO mice have not been fully characterized in the paper. As neural progenitor proliferation may impact neuronal fate determination and migration in the later stage, it is unclear if CAMDI-KO affects progenitor proliferation and fate specification. Authors should determine the proliferation of progenitors in the whole brain using BrdU labeling or other proliferation markers, such as Ki67 and phosphor-Histone H3 staining. Particularly, the authors have shown that CAMDI is highly expressed in the intermediate zone (THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 52,pp. 40554 -40561, 2010). Does CAMDI-KO affect intermediate progenitors? In page 7, the first paragraph line 4, the sentence "labeling progenitor neurons in the ventricular zone" is confusing. Do authors refer to progenitors or new-born neurons? The delayed migration of BrdU labeled neurons could be explained by slow proliferation. Since authors have done the BrdU labeling, is the overall proliferation rate changed by CAMDI-KO? In addition, to test if CAMDI plays a role in proliferation and cell fate determination, authors should quantify the total number of Cux1+ cells and measure other layer markers, such as foxp2.

> We agree that this point is particularly important for this study. The BrdU incorporation assay for labeling newborn neurons suggested that the overall proliferation rate did not change due to CAMDI KO (Figure EV2E). Consistently, the total numbers of Cux1-, CTIP2-, pHH3-, TBR2-positive neurons did not change due to CAMDI-KO (Figure EV2B-D). Thus, we conclude that delayed migration by CAMDI KO is not due to alterations in cell proliferation and cell fate determination. We mentioned this in the text (page 6, lines 10-15).

2. For all behavioral tests, the gender will significantly affect the results. The authors should indicate how many male and female mice are used in the method. They should do the analysis separately on male and female mice to determine which gender drives the behavioral difference. Moreover, the authors indicated in the methods "Mice were tested sequentially" in different behavioral tests. However, the figure captions seem to indicate that different numbers of mice were used in different experiments. Were these indeed different groups of mice, or were they the same groups, and different numbers used from each group? If the latter, why were some mice excluded from some experiments?

> In this study we used male mice in all behavioral studies as described in Materials and Methods (page 28, line 6). The meaning of "sequentially" is in order from low stress tests to high stress tests. To avoid misunderstanding, we removed this word. The number of mice used for the behavioral tests was indicated in each figure legend. The number of mice in each behavioral test was different because different groups of mice were used for the behavioral tests. We explain this in the M&M (page 28, lines 10-12).

3. In the open field test, the CAMDI-KO mice show the hyperactive behaviors with similar traveling time as WT mice. Does the open field test indicate CAMDI-KO mice more anxious? Do they spend less time in the center than WT mice?

> According to this comment, the time spent in the center of open field was assessed. As expected, CAMDI-KO mice spent less time in the center than WT mice (Figure EV3C). Since light-dark test also suggested an anxious phenotype of CAMDI-KO mice, we conclude that CAMDI-KO mice are more anxious than WT mice. We mentioned this in the text (page 8, lines 5 - 8).

4. In the social interaction test, it seems that there is a significant difference between the KO mice interacting with the strange mouse vs the empty cage, which would mean the KO mice still prefer the stranger to the empty cage, indicating that the KO mice show normal social behavior. This is not valid to claim a social defect if KO mice can still distinguish an empty cup versus a stranger mouse. Please run the statistics on this.

As the reviewer pointed out, statistics indicated a difference between the KO mice interacting with the strange mouse vs the empty cage (Figure 2D), indicating that CAMDI KO mice did not lose social interaction behavior. However, CAMDI-KO mice spent significantly less time interacting with the strange mouse than WT mice (Figure 2D, EV3G). Therefore, we used here "reduced social interaction behavior" not social defect. We explain this in the text (page 9, line 1- line 3).

5. It is interesting that CAMDI overexpression inhibited the HDAC6 activity and upregulated Î±-tubulin acetylation. Is CAMDI a general inhibitor of HDAC6 or specific to tubulin? Will this physical interaction block HDAC6 enzyme activity with other substrates? The centrosome abnormality will affect both proliferation and the migration. Many papers have shown that HDAC6 involves in multiple cellular events, including proliferation. Will tubastatin A treatment rescue the migration defect possibly through proliferation?

In order to examine whether CAMDI is a general inhibitor of HDAC6 or specific to Ac-tubulin, we assessed the level of acetylated HSP90, which is known to be a substrate for HDAC6 [20]. The immunoblot analysis demonstrated that the Ac-HSP90 level was not changed by CAMDI KO (Figure EV4C), suggesting that this physical interaction did not block HDAC6 enzyme activity with Ac-HSP90, and that CAMDI specifically inhibits HDAC6-mediated catalysis of Ac-tubulin. We mentioned this in the text (page 11, line 15-page 12, line 4).

Although HDAC6 has been shown to be involved in cell proliferation [22], there was no significant difference in the number of Cux1-positive neurons after treatment with or without Tubastatin A (Figure EV4E), suggesting that the rescue of delayed migration by Tubastatin A was not due to the change in cell proliferation. We mentioned this in the text (page 14, lines 1-5).

6. Please explain why not to rescue all behavioral defects, and why only select some behaviors for a rescue. It would make sense to include the social interaction test, as you claim before that deficits in this are part of the autism symptom triad.

Tubastatin A could rescue some behaviors, but not all behavioral defects such as social behaviors in CAMDI-KO mice. In this study, Tubastatin A was administered to pregnant mice only during E12.5–E17.5. Since social behaviors are generally acquired through various experiences after birth, they may not be rescued by recovered migration during the prenatal period. If Tubastatin A was administered continuously after birth, all behaviors may have been rescued. Alternatively, besides HDAC6 inhibition, CAMDI may have other function(s) such as dendrite formation and spine maturation in the adult stage, because CAMDI interacts with myosin regulatory light chain 2a. If so, not all behaviors might be rescued even if Tubastatin A was administered continuously after birth. We explained this in the Discussion (page 19, lines 4 – 13).

Minor Comments

1.Figure 1A seems oversaturated. Perhaps turn down the gain on the confocal?

According to this comment, we replaced the WT Cux1 staining of Fig 1A with better images.

2.Figure 1E, the WT brain section is clearly more rostral than the corresponding KO section. The author should compare sections at similar brain region.

According to this comment, we provided WT image at the same bregma level with KO image in Fig 1E. We believe that it becomes clear.

3.The authors mentioned that delayed cortical migration and aberrant axonal projections detected in CAMDI-KO brains are associated with psychiatric disorders, but did not cite any paper supporting this claim.

> Regarding the delayed cortical migration, we cited in the discussion (page 16, lines 4-7) (On the other hand, postmortem studies on the brains of patients with schizophrenia or autism as well as in mice with the responsible genes knocked-out demonstrate abnormal neuronal migration [35-40], indicating a causal relationship between psychiatric disorders and aberrant neuronal migration.)
>
> Regarding the aberrant axonal projections, we cited in the discussion (page 15, lines 12-14). (Indeed, corpus callosum thinning was observed in patients with ASD [24, 25], suggesting that long-distance disconnection is a pathophysiological state in ASD [26, 27].)

4.Page 42, figure 4 title, "Figure 4. HDAC6 inhibition by CAMDI regulates Î³-tubulin localization at the basal bodies". It should be Î±-tubulin.

> We are very sorry for this careless mistake. The title of this section was changed to “HDAC6 inhibition by CAMDI regulates Ac-Tubulin level at the basal bodies” (page 47, lines 14-15).

5.Since DISC1 interacts with CAMDI, will DISC1 modulate CAMDI mediated migration? This should at least be discussed.

> DISC1 has also been shown to accumulate in the centrosome and regulate centrosomal function as well as neuronal migration. Since we previously suggested that CAMDI translocates to the centrosome in a DISC1-dependent manner, it is possible that CAMDI and DISC1 coordinately regulate the centrosome stability during cortical migration. We mentioned this in the discussion (page 18, lines 8-12).

Referee #2:

In this study, Fukuda et al produce a CAMDI knockout mouse model to recapitulate neuronal migration phenotypes observed previously in a transient knockdown model. The authors also show that knockout mice exhibit hyperactivity, repetitive behavior, and social interaction deficits. To account for a mechanism to the observed findings, an interaction was described between CAMDI and HDAC6 and that loss of CAMDI resulted in reduced levels of g-tubulin and acetylated tubulin. Given these observation, the authors queried whether activation of HDAC6 might drive neuronal migration and behavioral phenotypes. Interestingly, treatment of mice with an inhibitor to HDAC6 rescued these phenotypes along with increasing protein levels of acetylated tubulin from brain cortical lysate. Over all, this is an interesting piece of work that will move the field forward. However, there are several limitations with the story and the logic is somewhat hazy in several parts of the manuscript.

1. Introduction. The authors make an argument using human genetics to indicate that CAMDI and DISC1 are relevant biological targets. Unfortunately, this is currently highly unlikely. The are several noteworthy publications questioning whether the initial Scottish DISC1 pedigree was analyzed correctly, and more importantly, the validity of DISC1 as a major/minor SZ candidate has not been shown to be true (case in point, the 108 loci paper and several others). With regards to CAMDI, the papers that the authors cite on DISC1 conditioned GWAS indicate that CAMDI (at a p-value=10E-4) is no different from an event occurring by chance. As such, I would focus on the facts in the introduction, rather than speculation (i.e. DISC1 is a major risk factor for psychiatric disorders - this is not a true statement).

> As pointed out by the reviewer, introduction about DISC1 was too speculative. We described only objective facts in the introduction (page 3, lines 6-12).

2. Behavioral tests. Over all, I find the approach to be appropriate, but the analyses are concerning. It appears that most comparisons are being performed using t-tests (which is fine for some comparisons) and I'm unable to get a sense of the "true" significance. If the authors are comparing 2

genotypes on the open field (and the other assays), I would like to have more information on the statistical analyses (F-values for interactions and main effects). Also, I'm unsure how long the animals have been placed in the OFT; in figure 2B, the total distance for adults is ~20m and in Figure 7b for juveniles, it is ~7m? Some other variable must be different here. Also, why is there a need to compare juveniles and adults? Is there a progressive behavioral and biochemical deficit? Finally, it appears that the mutants travel more distance in the perimeter (another indicator of anxiety)?

As pointed out by the reviewer, we performed one-way or two-way ANOVA analysis and included the statistical information of behavioral tests in each figure legend (F-values for interactions and main effects).
In the open field test, adult mice were placed for 15min and juvenile mice were placed for 10 min. This information was described in the M&M (page 29, line 12-13 and page 32, line 15) and figure legend.

Psychiatric behaviors in human are often different between juveniles and adults. Some juvenile behaviors disappear in adults, and the juvenile experiences affect on social behaviors in adults. In the open field test, CAMDI-KO mice showed an increase in travel time without a velocity change (Figure EV3A, B) and spent less time in the center of the field than WT mice (Figure EV3C), suggesting that CAMDI-KO mice are more anxious than WT mice. Interestingly, the behavioral differences between WT and CAMDI-KO mice become reduced in the adult stage. Similarly, the grooming time was comparable between WT and adult CAMDI-KO mice (Figure EV3D), suggesting progressive and age-dependent behavioral alterations. We mentioned this in the text (page 8, lines 5 - 11).

3. Two main points of hazy logic to me: a Y2H experiment was performed, but no information was provided about the details of the library (species of the library, tissue context, what CAMDI probe was utilized for the bait, and importantly, what fragment of HDAC6 was identified from the library). All of this information can be used to potentially bolster your specificity argument in Figure 3. The second point of confusion is the use of HeLa cells to show an effect of CAMDI and HDAC6 on g-tubulin and acetylated tubulin levels. The author indicate that knockout mice are healthy and viable with early migration and behavioral phenotypes. Why, now transition to a cervical cancer cell line? While the authors should be applauded for validating some findings using brain lysate, they should really consider a neuronal cell line at the very least (I noticed some information about SH-SY5Y cells in the methods, but nowhere else) for mechanistic details. In addition, conclusions from all blots and images should be quantified. The reader is presented with representative blots and immunohistochemistry, but has no way of understanding what has been repeated.

Information about a Y2H experiment was included in the revised manuscript (main text page 10, lines 6 – 8, and M&M). In this study we used HeLa cells in Fig 4A, 5A-D, EV4D for immunocytochemical analysis of centrosome and basal body localization because the cell line is generally used for analysis of cell cycle and centrosome. Indeed, centrosome was normally and clearly detected in HeLa cells. In addition, SH-SY5Y cells, a human neuroblastoma cell line, are not suitable for this experiment because cell body is very small and Ac-tubulin is aggregated. This is the reason why we used HeLa cells in this study. We explained this briefly in the result (page 11, lines 2-3).
According to this comment, we quantified all blots and images except for Fig. 3A and B. because IP:IB assay was not suitable for quantification. We performed at least 3 experiments and included the number of experiments in the figure legends.

4. Finally, the authors show a mild migration phenotype and possibly abnormal projection of neurons. It should be examined whether the mice show some more brain phenotypes relevant to psychiatric disorders, such as reduction of parvalbumin, perineuronal nets, oligodendrocyte markers, increase of the expression of inflammation related molecules, and alterations of neuronal morphology (such as fewer and/or smaller spines, poorer dendrites etc). Also, transcriptome or proteome analyses of some brain regions of the KO mice could be done and the results could be compared to those of the post-mortem brains of human patients.

To examine whether CAMDI-KO mice show few other brain phenotypes relevant to psychiatric disorders, we performed qRT-PCR to address the expression levels of TNF-a, IL-

1b, IL-6 (inflammation related molecules), and myelin basic protein (oligodendrocyte marker), and assessed the number of parvalbumin-positive cells. The TNF-a expression was increased in CAMDI-KO mice but other parameters were comparable to those of WT mice. (Figure EV5). We mention this in the discussion (page 16, lines 10 - 16).

We appreciate for reviewer's comments and suggestions about transcriptome or proteome experiments. We performed gene-chip analysis and identified very interesting alterations of various mRNA expression levels. We are currently investigating the molecular mechanism underlying these alterations in detail and preparing it as a next project. So, please forgive us for excluding this information at this time.

Minor points.

1. The authors mention southern probes, but don't show data

According to this comment, information of southern probe is included in M&M (page 22, lines 3-6).

2. Various typos throughout the manuscript (i.e. Page 9 "social interaction
test, the compared...")
3. Mol weights should be indicated on immunoblots.

We are sorry for our careless mistakes. All the text has been checked again and these mistakes were corrected. Molecular weights were indicated on immunoblots.

Referee #3:

EMBO Reports manuscript
"Rescue of CAMDI deletion-induced radial migration and psychiatric behaviors by HDAC6 inhibitor." By Fukuda et al. The authors' laboratory has previously identified CAMDI as an interactor of DISC1 and myosin II. A knock-down of CAMDI was shown to impair centrosome orientation and radial migration of cortical neurons. Here, the authors characterise a CAMDI knock-out mouse. The authors claim that CAMDI knock-out induces a delay in the cortical migration of Cux1+ neurons born at E14.5, which also mis-project to striatal regions. Further, CAMDI KO mice show behavioural abnormalities including social deficits and repetitive behaviours. To elucidate the molecular function, the authors show that CAMDI interacts with HDAC6, which is involved in tubulin acetylation. In overexpression studies, CAMDI and HDAC6 antagonise each other, which is taken as indication that CAMDI inhibits HDAC6. In vivo inhibition of HDAC6 rescues several defects of the CAMDI knock-out, including rescuing the mis-localisation of cortical neurons, overcompensates tubulin acetylation, and rescues several but not all behavioural abnormalities. In conclusion, this manuscript offers characterization of a new mouse model and shows interesting data to suggest that CAMDI has a role in tubulin acetylation. Several key conclusions of the paper, however, are not fully supported by the data, and therefore this study requires revisions in order to be acceptable for publication at EMBO reports. Points that should be addressed by the authors are as follows.

1.In Figure 1A, the authors show a clear mis-positioning of Cux1-positive cells in CAMDI KO mice at postnatal day 2. However, when quantifying the positioning of these cells, the authors perform a Student's t test to compare between the WT and KO distribution. When comparing distributions, Student's t test is not an appropriate test. The authors should perform an appropriate statistical test for this eg. two-way ANOVA. This statistical concern also applies to quantifications in Figure 1D and Figure 6B.

According to this comment, we performed Two-way ANOVA test for Fig1B, 1D and 6B. The summary information was included in each figure legend (page 43, lines 10-11, page 44, line 1-2, page 51, line 14-15).

2. In Figure 1C, the authors describe the mis-positioning of EGFP-positive neurons at P21 as 'delayed migration', which implies that the neurons will eventually reach their final destination.

However, since this time-point is at the end of brain development (P21), further migration unlikely and this rather suggests mis-localisation of neurons to the lower cortical layers. In order to confirm this, and to check if these neurons also assume the identity of the layer they are in, see the mis-projection of their axons, the authors should use further markers of cortical layering to characterise the fate of these mis-positioned neurons eg Cux1, Ctip2. Additionally, on page 8 first paragraph, the authors state that the mis-localised neurons in layer V had the 'properties of layer II/II neurons'. This appears refers to the fact that they are Cux1+ positive, which is in this reviewers' opinion insufficient evidence for this statement.

Mislocalization of neurons to the lower cortical layers in CAMDI-KO mice at P21 was further confirmed by other markers such as Cux1 and CTIP2 (Figure EV2A, page 6, lines 3-5).

3. In Figure 1E and Supplementary Figure 2, the authors show that CAMDI-KO mice present incorrect axon pathfinding, with neurons born at E14.5 projecting to the ipsilateral striatum. However the images shown in these figures appear not to be from the same Bregma level in the brain. To clarify this, the authors should provide images of WT and KO at the same bregma level, and clearly show/quantify the callosal projections in both WT and KO and the presence/absence of striatal projections. They should include information about the number of animals used for this analysis.

As suggested by the reviewer, we provided the WT image at the same bregma level with KO image in Fig 1E. We quantified the callosal projections in both WT and KO and the presence/absence of striatal projections (Figure 1F, EV2G-I, page 7, lines 8-9). Although no obvious difference was observed in the callosal projections, a significant difference was observed in striatal projection. We mentioned this and provided the number of mice (n=3 mice/genotype) used for this analysis in figure legends (page 44, lines 9-10 and page 57, line 2-6).

4.In the middle of page 7, the authors cite a shRNA knock-down of CAMDI. This appears to refer to their previous paper, which should be cited here; otherwise, it should be indicated that this data is 'not shown'.

We cited our previous paper here (page 6, line 9).

5. The authors use the open field test in Figure 2A-B to demonstrate locomotor hyperactivity in CAMDI KO. However more information on this test needs to be shown in order to exclude other conclusions that can be drawn from this test. The open field test can also be used to assess anxiety-like behaviors (thigmotaxis), and since the authors already detect an anxiogenic phenotype in the light-dark test (Figure 2I-J) they should show centre time in the open field to verify whether this phenotype is also detected in the open field (it is mentioned in the methods that time in center was measured, however it is not shown in results. This would help clarify whether their anxiety phenotype is interfering with the interpretation of the open field as a hyperactivity defect only. Additionally, in the result section for this figure (page 9), 'data not shown' is cited that the overall travel time remains unchanged. Since the travelled distance increases, does this mean that the mutant mice run faster? Would that really be hyperactivity, if the activity duration is unchanged?

CAMDI-KO mice showed an increase in travel time without a velocity change (Figure EV3A, B) and spent less time in the center of the field than WT mice (Figure EV3C), suggesting that CAMDI-KO mice are more hyperactive and anxious than WT mice. Interestingly, the behavioral differences between WT and CAMDI-KO mice become reduced in the adult stage. Similarly, the grooming time was comparable between WT and adult CAMDI-KO mice (Figure EV3D), suggesting progressive and age-dependent behavioral alterations. We mentioned this in the text (page 8, lines 5 - 11).

6. Further to point 5, in Figure 2D in the three-chamber test, the time CAMDI KO mice spent exploring the stranger mouse goes down but this does not result in more time spent interacting with the empty cage. The authors should show the total time spent in each chamber, including the neutral centre chamber, to clarify if the mice are actually showing preference for the neutral chamber over stranger mouse and an empty cage, or just spending time in the chambers of the stranger mouse and

empty cage without in fact interacting with them. The authors should also clarify in the methods sections what they define as "interaction time".

We showed the total time spent in each chamber including the neutral centre chamber in Fig. EV3G. No significant difference was observed between WT and KO mice. Interaction time means sniffing time as described in M&M (page 31, line 3). Although KO mouse spent time in the chambers of the stranger mouse, interaction time of KO mouse with the stranger mouse was reduced. Thus, we conclude that CAMDI KO mice show reduced social behavior. We explained this in the text (page 9, line 1-3).

7. The cage-hang test is taken as a measure for motor coordination (top of page9, supplementary figure S3). This test measures the strength of the motor units, not their coordination.

Thank you for pointing it out. This error was corrected in the text page (page 8, lines 14).

8. In Fig 3E, it should be indicated where the borders between the different fragments lie, to make the experiment easier to understand.

According to this comment, we indicated the border in Fig. 3E.

9. In Figure 3D, fragment 3 in the bottom panel seems to migrate faster than the other two fragments. This region of the gel is not shown in the top panel, and the reader cannot judge if this fragment binds CAMDI or not.

According to this comment, we replaced Fig 3D with better images.

10. Figure 4A is cited as indication that CAMDI overexpression increases tubulin acetylation. What is this compared to? There are only two non-transfected cells in the right periphery of the micrograph, and their acetyl-tubulin signal does not look so much weaker than that of the upper transfected cell.

To demonstrate enhanced tubulin acetylation by CAMDI overexpression, we indicated the ratios of Ac-tubulin intensity by line scanning along the line from immunocytochemical images (Fig 4A) (page 11, lines 1-2).

11. The Western blots in Fig. 4B-4D need to be quantified, and the significance of the effects should be evaluated with several biological replicates.

According to this comment, the Western blots in Fig. 4B-4D were quantified and indicated the number of experiments in the figure legends (page 48, line 6, 9 and 13). We examined whether CAMDI was a general inhibitor of HDAC6 or specific to Ac-tubulin. We assessed the level of acetylated HSP90, which is known to be a substrate for HDAC6 [20]. The immunoblot analysis demonstrated that the Ac-HSP90 level was not changed by CAMDI KO (Figure EV4C), suggesting that CAMDI specifically inhibits HDAC6-mediated catalysis of Ac-tubulin.

12. Figures 4 and 5 are taken as proof that CAMDI inhibits HDAC6. Instead, it is only shown that they antagonise each other: the data could also be explained if CAMDI attracts acetylation rather than inhibiting deacetylation. The interaction of the two proteins is not a proof of direct inhibition; biological complexes can sometimes bring two antagonising activities together. To show inhibition, it would be necessary to demonstrate that CAMDI has no effect on tubulin acetylation in the absence of HDAC6.

To further verify the specificity, we examined the effect of CAMDI on tubulin acetylation in the condition of HDAC6 knockdown. As expected, CAMDI had no effect on tubulin acetylation in the condition of HDAC6 knockdown (Figure EV4A, B). We mentioned this in the main text (page 11, lines 12-15).

13. In Figure 6A and B, the authors show that after Tubastatin A treatment they could rescue the neuronal mis-positioning of Cux1-positive neurons. To further confirm these results, it is

recommended that the authors analyze neuronal migration and axonal pathfinding after Tubastatin A treatment, which will better support their data and the fact that Tubastatin A can also rescue some of the behavioral phenotypes observed in CAMDI-KO mice.

> According to this comment, we challenged to analyze neuronal migration and axonal pathfinding after Tubastatin A treatment. We predict that the projection is cured if migration is normalized. However, CAMDI KO mice are vulnerability to stresses and they did not bring up children after Tubastatin A treatment during E12.5-17.5. Unfortunately, we could not overcome this problem in time. We would like to treat that as the next project.

14. In Figure 7D, the authors show that P21 CAMDI-KO mice exhibited decreased grooming time, while in adult mice there is no grooming phenotype (page 9, data not shown). This is intriguing and suggests that some aspects of the repetitive behavior phenotype seen in juvenile CAMDI-KO mice are age-dependent and are ameliorated by adulthood without treatment. The authors should comment on this.

> Psychiatric behaviors in human are often different between juveniles and adults. Some juvenile behaviors disappear in adults, and the juvenile experiences affect on social behaviors in adults. In the open field test, CAMDI-KO mice showed an increase in travel time without a velocity change (Figure EV3A, B) and spent less time in the center of the field than WT mice (Figure EV3C), suggesting that CAMDI-KO mice are more anxious than WT mice. Interestingly, the behavioral differences between WT and CAMDI-KO mice become reduced in the adult stage. Similarly, the grooming time was comparable between WT and adult CAMDI-KO mice (Figure EV3D), suggesting progressive and age-dependent behavioral alterations. We mentioned this in the text (page 8, lines 5 - 11).

15. The authors should comment in the discussion on why Tubastatin A might be improving hyperactivity, impulsive behavior and environmental adaptation but not social behavior in CAMDI-KO mice.

> Tubastatin A could rescue some behaviors, but not all behavioral defects such as social behaviors in CAMDI-KO mice. In this study, Tubastatin A was administered to pregnant mice only during E12.5–E17.5. Since social behaviors are generally acquired through various experiences after birth, they may not be rescued by recovered migration during the prenatal period. If Tubastatin A was administered continuously after birth, all behaviors may have been rescued. Alternatively, besides HDAC6 inhibition, CAMDI may have other function(s) such as dendrite formation and spine maturation in the adult stage, because CAMDI interacts with myosin regulatory light chain 2a. If so, not all behaviors might be rescued even if Tubastatin A was administered continuously after birth. We explained this in the Discussion (page 19, lines 4-13).

2nd Editorial Decision 02 September 2016

Thank you for the submission of your revised manuscript to our journal. We have now received the comments from all referees, copied below, and I am happy to tell you that all support its publication now. The paper can therefore in principle be accepted.

Please address the minor comments by referee 2. The panels in Fig 6a seem to be labeled twice, please check. When you upload the new manuscript, please upload the figures as individual files and move the EV figure legends to the end of the main manuscript file.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a new final version of your manuscript as soon as possible.

Referee #1:

This revision has addressed all the questions that reviewers have raised. Recommend to publish.

Referee #2:

The manuscript has improved significantly over the first iteration. While there are clearly some gaps in mechanism of why Tubastatin A can rescue some but not all phenotypes and why age plays a role in dissecting the phenotype, the authors have clearly produced a manuscript with exciting findings that have the potential to drive discovery in the field of neuroscience and therapeutics. My only minor comment is that the Y-axes could to be better described such that the reader does not have to turn to the figure legend for more information. For example, Figure 7D could be called grooming time, instead of time alone; or in EV3 D and E where both graphs are called Time (S), but really measure 2 different variables. Over all, a great job. In my humble opinion, this paper should be highlighted in a News and Views section

Referee #3:

The authors have extensively revised the manuscript with additional experiments.
I am happy with the revision, the paper should be published in its present form. It provides an important contribution to the fields of developmental neuroscience and intellectual disabilities.

2nd Revision - authors' response 06 September 2016

I am very excited to receive your decision letter that our manuscript can in principle be accepted. We corrected the following points in the final version.

The panels in Fig 6A labeled twice were checked and corrected.

According to the Referee's comments, we changed the label of Y-axes in each Figure as follows: Figure 7D (Grooming time), EV3D (Grooming time), EV3E (Olfactory time), EV3F (Cage-top hang time), respectively.

We greatly appreciate the reviewers' many helpful and constructive comments on our manuscript. Also, we truly appreciate you assigning such qualified reviewers.

I'm looking forward to hearing good news from you that our paper is highlighted in a News and Views section.

3rd Editorial Decision 12 September 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Shigeru Yanagi
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42416-T

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	For biochemical analysis, we performed 3–independent experiments. For behavior studies, we estimated the sample size based on power calculations from another psychiatric model.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies, our sample size are similar to those generally used in the field.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	None of the animals were excluded in our experiments.
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.	All samples wer randomly selected.
For animal studies, include a statement about randomization even if no randomization was used.	Mice were divided into groups randomly.
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.	Biochemical analysis were not blind.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Page 28, Mterials and methods
5. For every figure, are statistical tests justified as appropriate?	All figure legends have this information.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	Page 34, Mterials and methods and figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes.

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).	Page 22, Mterials and methods

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Page 24, Mterials and methods

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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.	Page 21, Mterials and methods
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Page 21, Mterials and methods
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.	I have confirmed.

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
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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
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