EMBO reports

A placental mammal-specific microRNA cluster acts as a natural brake for sociability in 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 22 June 2018

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think the manuscript is of interest, but requires further revisions to allow publication in EMBO reports. All three referees have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, and I think all the points need to be addressed, I will not detail them here. Please also provide the quantifications of the experiments as indicated by referees #1 and #3.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your 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.

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Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this

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- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures)

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:

Lackinger et al report the further analysis of a mutant mouse lacking the miR379-401 cluster that harbors 38 miRs linked to synaptic plasticity and neuropsychiatric diseases and that was reported before by the authors. Due to specific hints from the literature that the miR379-401 cluster may play a role in social behavior and related diseases, Lackinger et al specifically analyzed social behavior in the mutant mice. The data show that miR379-401 mice exhibit increases social behavior across lifespan. At the molecular level the authors provide evidence that loss of miR379-401 increases synaptic transmission and morphology (increases spines). Using gene-profiling a number of mRNA coding for key synaptic proteins such as glutamate receptors are identified as candidates that are up-regulated due to the loss of 5 miR of the miR379-401 cluster. Regulation of target genes by these miRs is confirmed in primary neurons.

The study reports interesting observations and provides first insight to potential underlying mechanisms. Especially the finding that loss of miR379-401 leads to enhances sociability in mice is interesting in light of the existing literature on human psychiatric diseases.

Comments:

1.
Regarding the behavior data. Is there a gender effect? This should be reported.

2.
RNA-seq. The analysis should be reported in greater detail in the methods. Some info is found in the methods, the text, supplement and figure legend. For example in the text the number of transcripts is reported on the basis of the FDR but the panel in Fig3a suggest that also a LOGFC cut off was applied for pathway analysis. Please clarify.

3.
RNA-seq was performed in adult mice which likely explains that most of the differentially expressed genes are not direct targets of the miR379-401 cluster that is lacking already from pre-natal developmental stages. One would hypothesize that the selected candidate miRs and mRNA targets are therefore already de-regulated early in the development and that the large amount of differentially expressed genes observed in adult animals represent secondary effects. It would thus be interesting to perform a similar experiment during development or alternatively select candidate genes and "adult de-regulated" non-candidate genes for qPCR. Another way to address this question could be to test if de-regulation of the candidate mRNAs could predict the massive gene-expression changes observed in adulthood.

4.
Considering the massive amount of mRNA differentially expressed and the related pathways linked to synaptic plasticity it is interesting that social behavior and anxiety is affected while other cognitive abilities appear to be unaffected. The authors should contrast their RNA-seq data with other studies that performed similar analysis form hippocampal tissue of mouse models that exhibit memory impairment to decipher differences and commonalities.

5.
N = 3 is a rather small experiment for RNA-seq but the data appears nevertheless sound and suggest that the gene-expression changes may even be underrepresented. It would be helpful to show a PCA in addition

6.
- typo page 5 for reference "13"
- Fig2e should be enlarged

Referee #2:

The authors explore the behavioral phenotype of the miR-379-410 mouse knockout and observe a very interesting hypersocial effect. This imprinted locus appears to be fairly recently evolved in mammals and is important for synaptic function and psychiatric illness. The authors claims are supported by in a number of behavioral paradigms, excitatory electrophysiology, dendritic morphology and molecular analyses.

Minor comments:

The introduction seems to be truncated and did not really setup the rest of the manuscript in relation to hypothesis, aims and brief outcomes. I was left feeling a little cold at this point.

Some basic information on the model and how it was generated would save the readers finding another paper to understand this. I think this is important given the unusual heterozygous targeting of the maternal chromosome. The mirg gene knockout was validated by in situ hybridization, but it

would be more convincing to see the impact of this knock out on the transcript or the mature miRNA. It is possible that there is some leakage from the paternal chromosome despite imprinting. How was the WT/KO genotyping performed? There was no description of this.

Barak and Feng 2016 - reference is not present in the reference list

An increase in sociability and anxiety-associated behaviours, coupled with intact memory function, suggests the miR379-410 KO may have more impact on brain regions other than the hippocampus, such as the mPFC or amygdala? In a study by Hollins et al 2014 (PMID:25268256) alteration of the miR-379-410 miRNA cluster was observed in the entorhinal cortex in rats exposed to polyIC and adolescent cannabinoid. This paper should be references in relation to discussion about the region of influence for the miR-379-410 miRNA cluster. Considering the extent of miR379-410 regulation of genes associated with synaptic function in the hippocampus, is there any possible explanation to why memory function was intact?

Is there any studies on miR-379-410 gain-of-function effects? While some reference was made to Angelman's syndrome, some more discussion about the human deletion syndromes in the syntenic locus would be interesting.

The legends for Fig. S3a-c and Fig. S3d seem to me mixed up.

Considering previous research from the group, is it possible that the behavioural phenotype is solely due to downregulation of miR-134 in this model?

Why were these interactions investigated using luciferase reporter gene in primary rat cortical neurons instead of mouse cells? Perhaps a note on the reasoning should be added to the methods.

It would be interesting to see if miRNAs with similar seed sequence residing outside of the miR379-410 locus are upregulated, as a compensatory/homeostatic response to miR-379-410 loss?

The authors suggest that "enhanced excitation in miR379-410 ko neurons could be the result of inefficient homeostatic compensation." This is an interesting hypothesis which could be explored by an expression time course for the miR379-410 cluster miRNAs after excitatory signalling to determine the extent (if any) of homeostatic compensation.

In figure 4a, "X" would seem to refer to the number of binding sites (not clear) and "*) denotes wobble interactions, it would be nice to also be able to see the category of binding site from TargetScan.

Referee #3:

The manuscript by Lackinger et al. investigates the role of the miR379-410 microRNA cluster in regulating social behaviors and demonstrates that loss of this cluster leads to increased sociability and anxiety-like behavior in mice. The miR379-410 cluster is a maternally imprinted miRNA cluster that encompasses >40 miRNAs. Changes in miR379-410 expression have been recently linked to psychiatric diseases such as schizophrenia and autism. The authors employ a genetic approach to address the role of the miR379-410 cluster in regulation of social behavior in mice. Using a constitutive deletion of the miR379-410 cluster, the authors perform a thorough behavioral analysis in wild type and mutant mice demonstrating that the miR379-410 cluster is a key regulator of sociability. The behavioral findings are associated with an increase in neuronal EPSC frequency and spine density as well as an increased expression of genes involved in synaptic transmission in the hippocampus. These findings suggest a possible link between the behavioral phenotypes and the increased excitatory synaptic drive in the hippocampus of miR379-410 deficient mice.

The manuscript is well written and easy to follow. The major strength of the paper is the strong impact of miR379-410 on social behavior and it's therapeutic potential, the major weakness is the lack of mechanistic insight into how miR379-410 exerts its control. The carefully drafted discussion of the potential role of this microRNA cluster in disease states, which involves both hypo- and

hyper-socialization phenotypes, invites the reader to think about this microRNA cluster as having an inverse relationship with sociability. In this scenario, the level of miR379-410 cluster expression could directly determine the level of sociability in mice and other mammals. While a defined level of miR379-410 expression would govern normal social behaviors, large deviations could trigger various disease states associated with abnormal sociability. However, while exciting, the manuscript has several shortcomings that have to be addressed. In terms of trying to define a mechanism by which the miR379-410 cluster regulates sociability, the manuscript would greatly benefit from any further steps taken to make the molecular analyses more direct in nature and to demonstrate changes in post-transcriptional regulation of proposed target genes (protein expression changes by Western, IF or other methods) in response to changes in the miR379-410 cluster.

Figure 3: The authors' approach of using RNA-seq and then performing motif analysis of upregulated DEGs remains rather correlative in nature. In order to identify the direct miR-379-410 regulated target genes in vivo, the authors could assess which of the predicted gene targets shows a loss/reduction in Argonaute2/RISC complex association upon -379-410 cluster ablation. It would be helpful if the authors could validate that the increased expression of some of the proposed target mRNAs (and implied decreased incorporation into the RISC complex) does indeed result in increased mRNA translation/protein expression by using Western Blot analysis and/or IF.

b-c: While the GO term enrichment analysis presented is informative regarding how the transcriptome of the hippocampus changes in response to loss of the miR-379-410 miRNAs, it would be more relevant to the specific function of the cluster to include a GO term enrichment analysis specifically for those upregulated DEGs that are predicted to be targets of the miR-379-410 cluster based on the motif analysis performed. Such an analysis would allow the authors to more specifically discuss particular pathways, etc. that the miR-379-410 miRNAs potentially directly regulate and avoids conflating indirect, possibly compensatory changes with the regulatory activity of the miR-379-410 miRNAs.

Figure 4d: The data presented do not strongly support the authors' conclusion that these ionotropic glutamate receptor components are direct targets of the miR-379-410 miRNAs. One would expect mutating the 3'UTR of the target genes would block the decrease in their luciferase activity following PTX treatment similarly to the decrease demonstrated with directly blocking the miRNAs via "anti-miRNAs." However, none of the control treated mutated 3'UTR conditions seem to be significantly increased as compared to the control treated WT 3'UTR condition (with the exception of perhaps Prr7) leaving the reader to wonder if the effect is a more general result of transfection with an "anti-miRNA," particularly since no unrelated miRNA was included to demonstrate miRNA specificity.

Minor criticisms:
Figure 2a.
Images are over saturated

Figure S5.
The claim that there is no change in dendritic complexity should be accompanied by a Sholl analysis particularly because the images actually seem to suggest an increase in dendritic arborization upon loss of the miR-379-410 miRNAs.

Figure 3.
- While the authors have provided evidence in Figure 2a-b to show the neuronal specificity of miRNA expression, similar measures are needed to demonstrate neuronal specificity in ex vivo tissue at least in the hippocampus. It would greatly strengthen the authors' interpretation and discussion of the RNA-seq data.

- Ages of mice used for the RNA-seq analysis are needed.

Figure 4
b. Ages of mice used for analysis needed.

c-d.

- The luciferase assays was performed in rat cortical neurons while all other molecular and electrophysiological work was done in hippocampal cells, please explain.
- Data validating the level of expression for each mimic used is needed, particularly because an unrelated mimic (miR-495) was used to demonstrate miRNA specificity. Without this validation, one may speculate whether these effects are due to these specific miRNAs or if, for example, transfection with the miR-495 mimic was not as efficient.
- It is unclear what the controls used in all the assays were. Please define more directly.

Figure S8.
b. Assay results with the unrelated mimic must be shown for Cnih2 and Scr, as well.

Methods.
Luciferase assay. 4 data points are shown per condition in the results; however, methods indicate transfections were performed in triplicates. Do data points represent average of technical triplicates for four independent experiments? Please define.

Statistical Analysis. Authors must indicate which posthoc test was used following 1Way ANOVA analyses.

1st Revision - authors' response 2 October 2018

Response Letter: Lackinger et al, EMBOR

Referee #1:

Lackinger et al report the further analysis of a mutant mouse lacking the miR379-401 cluster that harbors 38 miRs linked to synaptic plasticity and neuropsychiatric diseases and that was reported before by the authors. Due to specific hints from the literature that the miR379-401 cluster may play a role in social behavior and related diseases, Lackinger et al specifically analyzed social behavior in the mutant mice. The data show that miR379-401 mice exhibit increases social behavior across lifespan. At the molecular level the authors provide evidence that loss of miR379-401 increases synaptic transmission and morphology (increases spines). Using gene-profiling a number of mRNA coding for key synaptic proteins such as glutamate receptors are identified as candidates that are up-regulated due to the loss of 5 miR of the miR379-401 cluster. Regulation of target genes by these miRs is confirmed in primary neurons.

The study reports interesting observations and provides first insight to potential underlying mechanisms. Especially the finding that loss of miR379-401 leads to enhances sociability in mice is interesting in light of the existing literature on human psychiatric diseases.

We appreciate that this reviewer finds our study interesting in the light of the existing literature on human psychiatric disease.

Comments:

1.
Regarding the behavior data. Is there a gender effect? This should be reported.
> sexes have been reported separately (add one sentence in the discussion)

We agree with this reviewer that the reporting of potential gender effects is important. In this regard, we would like to point out that for all the behavioural experiments (Fig. 1, Fig. EV1, Appendix Fig-3), data was already presented separately for females and males. Thereby, we did not detect any differences between the sexes except for the elevated plus maze (EPM) test (Fig. EV1b), where the genotype effect is mainly in females. A detailed statistical assessment of the behavioural data with respect to gender differences is presented in our new Appendix file.

2.
RNA-seq. The analysis should be reported in greater detail in the methods. Some info is found in the

methods, the text, supplement and figure legend. For example in the text the number of transcripts is reported on the basis of the FDR but the panel in Fig3a suggest that also a LOGFC cut off was applied for pathway analysis. Please clarify.
As suggested by this reviewer, we have now described RNAseq experiments in more detail in the respective methods section. For the panel in Fig. 3a and all subsequent analysis of differentially expressed genes, no LOG fold change cut-off was applied for pathway analysis.

3.
RNA-seq was performed in adult mice which likely explains that most of the differentially expressed genes are not direct targets of the miR379-401 cluster that is lacking already from pre-natal developmental stages. One would hypothesize that the selected candidate miRs and mRNA targets are therefore already de-regulated early in the development and that the large amount of differentially expressed genes observed in adult animals represent secondary effects. It would thus be interesting to perform a similar experiment during development or alternatively select candidate genes and "adult de-regulated" non-candidate genes for qPCR. Another way to address this question could be to test if de-regulation of the candidate mRNAs could predict the massive gene-expression changes observed in adulthood.

We agree with this reviewer that many differentially expressed genes identified in the adult hippocampus might not represent direct target genes but rather secondary changes due to the prolonged lack of miR379-410 miRNAs during the animal's development. However, we would like to point out that the validation of potential targets (except for Shank3) by qPCR (Fig. 4b) was done with hippocampal RNA from juvenile animals, thereby reducing the potential risk for secondary effects. Accordingly, all of the four candidates (Prr7, Src, Cnih2, Dlgap3) that were selected for follow-up validation by luciferase assays could be validated as direct miR379-410 targets (Fig. 4 c,d, Fig. EV4). As suggested by this reviewer, we have now also looked at the expression of an "adult de-regulated, non-candidate gene", Shank3, by qPCR in both juvenile and adult hippocampus. In agreement with Shank3 being a secondary target, its expression is only significantly different between wt and miR379-410 ko mice in adult, but not in juvenile mice (Appendix Fig. S9). In contrast, the potential direct target Cnih2 remains differentially expressed in adult mice (Appendix Fig. S9), although the respective data turned out to be too variable to reach statistical significance.

4.
Considering the massive amount of mRNA differentially expressed and the related pathways linked to synaptic plasticity it is interesting that social behavior and anxiety is affected while other cognitive abilities appear to be unaffected. The authors should contrast their RNA-seq data with other studies that performed similar analysis form hippocampal tissue of mouse models that exhibit memory impairment to decipher differences and commonalities.

As suggested by this reviewer, we have now screened the relevant literature, and found a couple of knockout models where cognitive performance was assessed along with gene expression profiling in the hippocampus (Kennedy et al., 2016; Jaitner et al., 2016; Aurajo et al., 2017). Unfortunately, these data are rather heterogeneous and it turned out to be impossible to deduce pathways that are commonly deregulated in these models and might not be represented in our model. Nevertheless, it is worth mentioning that none of the candidate genes that were investigated in more detail in the above cited studies (Hdac2, Adra2a, Penk, Htr5b, Foxp1) is differentially expressed in our model, consistent with different pathways responsible for the control of cognition and sociability. One hypothesis that is emerging from recent studies (Fanselow&Dong, 2010; Felix-Ortiz&Tye, 2014) is that in particular the ventral part of the hippocampus might be involved in the processing of social information, whereas the dorsal part is mostly responsible for cognitive processing. According to this model, one might expect sub-region specific differences in expression between models showing social vs. cognitive impairments. To address this, we plan to perform more detailed RNA profiling in specific hippocampal sub-regions, possibly at single-cell resolution in the future. This however constitutes an entire new project and is therefore in our opinion beyond the scope of the present study.
5.
N = 3 is a rather small experiment for RNA-seq but the data appears nevertheless sound and suggest

that the gene-expression changes may even be underrepresented. It would be helpful to show a PCA in addition

We agree that n=3 is the minimum number of independent biological replicates, but due to the high technical quality of our experimental pipeline, we nevertheless obtained highly reproducible data. A high number of differentially expressed genes were identified with high confidence (applying stringent statistics with genome-wide corrections for multiple testing) and subsequently validated by multiple different assays (qPCR, luciferase assays, Western blot).
Furthermore, we have added the requested principal component analysis (PCA; Appendix Fig. S6). From this plot, the expected segregation of biological replicates into "control" and "miR379-410 ko" conditions is clearly visible.

6.
- typo page 5 for reference "13"
- Fig2e should be enlarged

We thank the reviewer for pointing this out. The typo has now been corrected and Fig. 2e has been enlarged.

Referee #2:

The authors explore the behavioral phenotype of the miR-379-410 mouse knockout and observe a very interesting hypersocial effect. This imprinted locus appears to be fairly recently evolved in mammals and is important for synaptic function and psychiatric illness. The authors claims are supported by in a number of behavioral paradigms, excitatory electrophysiology, dendritic morphology and molecular analyses.

We are pleased that this reviewer speaks of very interesting effects that are supported by a number of analyses.

Minor comments:

The introduction seems to be truncated and did not really setup the rest of the manuscript in relation to hypothesis, aims and brief outcomes. I was left feeling a little cold at this point.

Following the suggestion of this reviewer, we have now added a paragraph to the introduction covering the hypothesis, aims and brief outcomes of the manuscript. Overall, we paid particular attention that all the relevant topics (social behaviour, neural development, microRNA, miR379-410) covered in the main part of the manuscript are sufficiently introduced including the most relevant literature. However, owing to space limitations, we were unable to extensively discuss individual topics and cite all potentially relevant literature.

Some basic information on the model and how it was generated would save the readers finding another paper to understand this. I think this is important given the unusual heterozygous targeting of the maternal chromosome.

In order to make it easier for the reader to understand the genetics, we have now included a new figure (Appendix Fig. S1) with the gene targeting strategy of the miR379-410 locus and a scheme of the breeding strategy to obtain miR379-410 knockout animals.

The mirg gene knockout was validated by in situ hybridization, but it would be more convincing to see the impact of this knock out on the transcript or the mature miRNA.

Following the suggestion of this reviewer, we have now more extensively characterized the model with respect to the expression of precursor and mature miRNAs in neonatal, juvenile and adult animals using qPCR (Fig. EV2). Thereby, we demonstrated that there was a complete absence of expression for multiple members of the miR379-410 cluster at the level of pre- and mature miRNA. In addition, the expression of multiple neuronal miRNAs that are expressed from different genomic

loci (miR-124, -132, -138) did not significantly change between WT and mutant animals, arguing against any compensatory response in the absence of the cluster.

It is possible that there is some leakage from the paternal chromosome despite imprinting.

Based on all the evidence we gathered from multiple assays (RNAseq, qPCR, FISH), there is negligible leakage from the paternal chromosome. Therefore, heterozygous mice that inherit the mutant allele from the mother can be considered as full knockout, in agreement with previous studies that employed a very similar model (Marty et al., 2016; Labialle et al., 2014).

How was the WT/KO genotyping performed? There was no description of this.

The genotyping of the mice has been already described in the initial presentation of the model and is outlined in fig S1(Valluy et al., 2015).

Barak and Feng 2016 - reference is not present in the reference list

We apologize for this mistake. The reference has now been added to the list.

An increase in sociability and anxiety-associated behaviours, coupled with intact memory function, suggests the miR379-410 KO may have more impact on brain regions other than the hippocampus, such as the mPFC or amygdala? In a study by Hollins et al 2014 (PMID:25268256) alteration of the miR-379-410 miRNA cluster was observed in the entorhinal cortex in rats exposed to polyIC and adolescent cannabinoid. This paper should be references in relation to discussion about the region of influence for the miR-379-410 miRNA cluster.

According to the suggestion of this reviewer, we have now introduced the reference Hollins et al. in the paragraph (p. 9) where we discuss a potential contribution of brain areas other than the hippocampus (e.g. amygdala, prefrontal or entorhinal cortex). In general, region-specific miR379-410 ko (using stereotactic injection of rAAV-Cre) will be performed in the future to pinpoint the relevant brain areas/circuitries.

Considering the extent of miR379-410 regulation of genes associated with synaptic function in the hippocampus, is there any possible explanation to why memory function was intact?
Although speculative at this point, one possible explanation could be that miR379-410 dependent regulation of targets is particularly important in the ventral hippocampus, which is involved in the processing of social cues (in contrast to the dorsal hippocampus which is more relevant for cognitive processing). In line with this, miR379-410 microRNAs show a trend towards higher expression in the ventral compared to the dorsal hippocampus (Marty et al., 2016). Please see also our response to Reviewer 1, point 4.

Is there any studies on miR-379-410 gain-of-function effects? While some reference was made to Angelman's syndrome, some more discussion about the human deletion syndromes in the syntenic locus would be interesting.

We have now added some more discussion about a potential association of deletions/duplications within the syntenic genomic region of human chr. 14q32.2 with neurodevelopmental defects (p. 9). These include uniparental disomy 14 (upd14) syndromes, such as Kagami-Ogata- and Temple-Syndrome, as well as copy number variations (CNVs) found in autistic patients and listed in the Sfari database.

The legends for Fig. S3a-c and Fig. S3d seem to me mixed up.
Thanks for pointing this out, the mistake has now been corrected.

Considering previous research from the group, is it possible that the behavioural phenotype is solely due to downregulation of miR-134 in this model?
Our bioinformatics analysis of RNAseq data in fact argue for an important contribution of miR-134 to the observed phenotypes, although other miRNAs (miR-485, miR-381, etc.) likely also contribute. The contribution of individual miRNAs will have to be tested in future experiments, e.g. by expressing individual miRNAs in the context of the cluster deletion (rescue) or by knocking out

individual miRNAs using Crispr/Cas9 technology, which is however beyond the scope of the present study.

Why were these interactions investigated using luciferase reporter gene in primary rat cortical neurons instead of mouse cells? Perhaps a note on the reasoning should be added to the methods.
We have more than ten years of experience with the culture of rat primary embryonic neurons (both cortical and hippocampal). Therefore, these cultures are very robust over longer periods of time (up to 4 weeks) and can be very reliably transfected. Recently, we also started to culture primary mouse neurons from different mouse mutants, but the health status of these cultures is still variable, so that we prefer rat neurons for standard experiments. Since both the cluster miRNAs and the target genes are 100% conserved between mouse and rat, we are convinced that results obtained from rat cultures are meaningful with regard to the mechanism underlying the behavioural and cellular phenotypes observed in the mouse model.

It would be interesting to see if miRNAs with similar seed sequence residing outside of the miR379-410 locus are upregulated, as a compensatory/homeostatic response to miR-379-410 loss?
We did not observe any significant upregulation of miRNAs outside the cluster in miR379-410 ko hippocampus based on qPCR (Fig. EV2). See also our response above. Besides, we are not aware of any miRNA families outside this locus that share seed sequences with miR379-410 members. There is however a large degree of redundancy between different miRNAs expressed from the miR379-410 locus, which allows to group them in families (Seitz et al., 2003). Other members (e.g. miR-134) however do not have “relatives” within or outside the cluster.

The authors suggest that "enhanced excitation in miR379-410 ko neurons could be the result of inefficient homeostatic compensation." This is an interesting hypothesis which could be explored by an expression time course for the miR379-410 cluster miRNAs after excitatory signalling to determine the extent (if any) of homeostatic compensation.
We apologize for being unprecise here, but according to our hypothesis, the lack of miR379-410 could lead to inefficient homeostatic regulation of miR379-410 target genes (rather than the microRNAs themselves), which in turn could result in a failure of homeostatic synaptic plasticity (i.e. inefficient downscaling of excitatory synapses, as observed under conditions of miR-134/485 blockade in primary hippocampal neurons (Fiore et al., 2014; Cohen et al., 2011)). In agreement with this hypothesis, we observe an upregulation of excitatory synaptic genes in the miR379-410 knockout hippocampus (Fig. 3). Many of these targets are further downregulated by chronic excitation induced by 48 hour picrotoxin (PTX) treatment in cultured neurons (e.g. Cnih2, Src, Prr7 (Fig. 4d), AMPA-R subunits (RNAseq data), etc.). The downregulation in wt neurons correlates with a persistent PTX-mediated upregulation of miR379-410 miRNAs (Fiore et al., 2014, Rajman et al., 2017).

In figure 4a, "X" would seem to refer to the number of binding sites (not clear) and "*) denotes wobble interactions, it would be nice to also be able to see the category of binding site from TargetScan.
The missing TargetScan information has now been added to the respective figure.

Referee #3:

The manuscript by Lackinger et al. investigates the role of the miR379-410 microRNA cluster in regulating social behaviors and demonstrates that loss of this cluster leads to increased sociability and anxiety-like behavior in mice. The miR379-410 cluster is a maternally imprinted miRNA cluster that encompasses >40 miRNAs. Changes in miR379-410 expression have been recently linked to psychiatric diseases such as schizophrenia and autism. The authors employ a genetic approach to address the role of the miR379-410 cluster in regulation of social behavior in mice. Using a constitutive deletion of the miR379-410 cluster, the authors perform a thorough behavioral analysis in wild type and mutant mice demonstrating that the miR379-410 cluster is a key regulator of sociability. The behavioral findings are associated with an increase in neuronal EPSC frequency and spine density as well as an increased expression of genes involved in synaptic transmission in the hippocampus. These findings suggest a possible link between the behavioral phenotypes and the increased excitatory synaptic drive in the hippocampus of miR379-410 deficient mice.

The manuscript is well written and easy to follow. The major strength of the paper is the strong impact of miR379-410 on social behavior and it's therapeutic potential, the major weakness is the lack of mechanistic insight into how miR379-410 exerts its control. The carefully drafted discussion of the potential role of this microRNA cluster in disease states, which involves both hypo- and hyper-socialization phenotypes, invites the reader to think about this microRNA cluster as having an inverse relationship with sociability. In this scenario, the level of miR379-410 cluster expression could directly determine the level of sociability in mice and other mammals. While a defined level of miR379-410 expression would govern normal social behaviors, large deviations could trigger various disease states associated with abnormal sociability. However, while exciting, the manuscript has several shortcomings that have to be addressed. In terms of trying to define a mechanism by which the miR379-410 cluster regulates sociability, the manuscript would greatly benefit from any further steps taken to make the molecular analyses more direct in nature and to demonstrate changes in post-transcriptional regulation of proposed target genes (protein expression changes by Western, IF or other methods) in response to changes in the miR379-410 cluster.
We are grateful for the overall very positive comments of this reviewer and have made extensive efforts to strengthen the dataset related to the regulation of target genes at the protein level (see below for more details).

Figure 3: The authors' approach of using RNA-seq and then performing motif analysis of upregulated DEGs remains rather correlative in nature. In order to identify the direct miR-379-410 regulated target genes in vivo, the authors could assess which of the predicted gene targets shows a loss/reduction in Argonaute2/RISC complex association upon -379-410 cluster ablation.
We agree with this reviewer that performing comparative Ago-CLIP experiments in the mouse hippocampus *in vivo* would represent a very powerful technology to differentiate between direct and indirect targets. In fact, we are currently establishing such experiments in the context of a different miRNA mutant mouse model, and already obtained preliminary results from RNAseq. However, our current protocol did not faithfully identify already validated targets of this specific microRNA, suggesting that it needs further optimization. One potential reason could be that the sensitivity of such an assay is rather low, since most of the target mRNAs will not be exclusively targeted by the miRNA of interest, but rather by a combination of multiple miRNAs. Therefore, lack of a single miRNA or a group of related miRNAs (as for example in the case of the miR379-410 ko) would not be expected to lead to dramatic differences in Ago pull-down efficiencies for most of the presumptive target genes between miRNA mutant and control. Taken together, we feel that comparative Ago-CLIP experiments are currently beyond the scope and that candidate approaches (e.g. luciferase reporter assays and Western blots performed in this study) can alternatively provide rather strong evidence for the existence of miRNA-target interactions under physiological conditions.

It would be helpful if the authors could validate that the increased expression of some of the proposed target mRNAs (and implied decreased incorporation into the RISC complex) does indeed result in increased mRNA translation/protein expression by using Western Blot analysis and/or IF.
Following the suggestion of this reviewer, we have now performed Western blot analysis for the two strongest candidates (Cnih2, Prr7) based on luciferase assay using hippocampal lysates derived from multiple ko and control animals (Fig. EV5, Appendix Fig. S11-12). Thereby, consistent with our results from RNAseq, qPCR and luciferase, we observed a significant upregulation of Prr7 (Fig. EV5) protein expression in the miR379-410 ko hippocampus. In contrast, Cnih2 protein was not differentially expressed in the hippocampus of WT and KO mice, which was likely due to the high inter-animal variability we observed for this protein. We consider several possible explanations for this variability: First, Western blot sensitivity is much lower compared to RNA-based technologies (qPCR, RNAseq), making it more difficult to pick up subtle differences in protein expression as those regularly seen for miRNA-dependent control. Second, whole hippocampal lysates contain proteins from different cellular origin (e.g. neuron, glia, blood cells, etc.), raising the possibility that cell-type specific changes (e.g. if miRNA-target regulation only takes place in neurons) could be masked by large amounts of “unregulated” protein present in other cell types. Finally, in neurons, changes in protein expression, in particular those for synaptic proteins, might manifest only at the subcellular level (e.g. in the synapto-dendritic compartment), given the fact that many of the miR379-410 members are enriched in neuronal dendrites. In the future, more careful examination of local protein expression will be performed, e.g. by Western blot analysis in synaptosomes and/or immunofluorescence in hippocampal slices.

b-c: While the GO term enrichment analysis presented is informative regarding how the transcriptome of the hippocampus changes in response to loss of the miR-379-410 miRNAs, it would be more relevant to the specific function of the cluster to include a GO term enrichment analysis specifically for those upregulated DEGs that are predicted to be targets of the miR-379-410 cluster based on the motif analysis performed. Such an analysis would allow the authors to more specifically discuss particular pathways, etc. that the miR-379-410 miRNAs potentially directly regulate and avoids conflating indirect, possibly compensatory changes with the regulatory activity of the miR-379-410 miRNAs.
We agree with the argumentation of the reviewer and have now included the requested refined GO term analysis in Appendix Figure S8.

Figure 4d: The data presented do not strongly support the authors' conclusion that these ionotropic glutamate receptor components are direct targets of the miR-379-410 miRNAs. One would expect mutating the 3'UTR of the target genes would block the decrease in their luciferase activity following PTX treatment similarly to the decrease demonstrated with directly blocking the miRNAs via "anti-miRNAs." However, none of the control treated mutated 3'UTR conditions seem to be significantly increased as compared to the control treated WT 3'UTR condition (with the exception of perhaps Prr7) leaving the reader to wonder if the effect is a more general result of transfection with an "anti-miRNA," particularly since no unrelated miRNA was included to demonstrate miRNA specificity.
We agree with the points raised by the reviewer but think that they can be addressed by clarifying the interpretation of the results of the luciferase assays presented in Fig. 4d. First, we would like to point out that, in contrast to the statement of the reviewer, the “CTR” condition in Fig. 4d is actually a condition which is transfected with an anti-miRNA with a sequence that is not supposed to target any cellular miRNA (Exiqon negative control A, sequence unfortunately not provided by the company). Therefore, all effects observed with the target anti-miRNAs are actually **sequence-specific**. Second, we have now performed a direct comparison of the wt and mut UTR conditions transfected with “CTR” anti-miRNA. This revealed a significant difference only for Prr7, but not for Src and Cnih2, suggesting that the PTX-regulation of Prr7 is strictly dependent on miR-329/-495, whereas miR-485 only partially contributes to the regulation of Cnih2 and Src (Fig. 4d). This observation is in agreement with miRNA binding sites predicted by Target Scan: whereas the miR-495 and miR-329 sites are the only conserved binding sites within the relatively short 3’UTR of Prr7, Src/Cnih2 in addition to miR-485 contain respectively 14/4 conserved binding sites for non miR379-410 miRNAs, many of which are expressed in neurons. In fact, the difference between wt and miR-485 mut UTR under control conditions seems to be the least for the Src 3’UTR, consistent with the presence of many additional functional miRNA binding sites. Importantly however, inhibition of cognate miRNAs **in all cases** specifically elevates expression of wt, but not mut UTRs, providing strong evidence for an inhibitory function of the respective endogenous miRNAs via interaction with these sites.

Minor criticisms:
Figure 2a.
Images are over saturated
We deliberately oversaturated the MAP2 signal in order to visualize the entire neuronal morphology, including fine dendritic branches.
Figure S5.
The claim that there is no change in dendritic complexity should be accompanied by a Sholl analysis particularly because the images actually seem to suggest an increase in dendritic arborization upon loss of the miR-379-410 miRNAs.
A quantitative analysis of dendritic complexity in miR379-410 ko compared to wt hippocampal pyramidal neurons based on Sholl analysis has already been provided in Valluy et al., Nat. Neurosci. 2015. This careful analysis of multiple neurons derived from independent animals did not reveal a statistically significant difference between the wt and miR379-410 condition.

Figure 3.
- While the authors have provided evidence in Figure 2a-b to show the neuronal specificity of miRNA expression, similar measures are needed to demonstrate neuronal specificity in ex vivo tissue at least in the hippocampus. It would greatly strengthen the authors' interpretation and discussion of the RNA-seq data.

FISH against miR-134 demonstrating high expression of this miRNA in mouse CA3 hippocampal neurons has been previously published (Jimenez-Mateos et al., 2012). Furthermore, the expression of the miRNA host gene Mirg has been documented by in situ hybridization in the Allen Brain Atlas project (see also our reference in the introduction):
http://mouse.brain-map.org/experiment/show/71212422
Intriguingly, within the hippocampus formation, Mirg expression appears to be restricted to principal neurons of the CA1 and CA3 region (but not dentate gyrus) as well as scattered cell bodies within the stratum radiatum and pyramidale, possibly representing interneurons. Together with our extensive characterization of primary hippocampal neurons, this suggests that expression of miRNAs from the miR379-410 is largely restricted to excitatory and inhibitory neurons.

- Ages of mice used for the RNA-seq analysis are needed.
The age of the mice has now been provided (p. 16).

Figure 4
b. Ages of mice used for analysis needed.
The age of the mice has now been provided in the respective figure legends.

c-d.
- The luciferase assays was performed in rat cortical neurons while all other molecular and electrophysiological work was done in hippocampal cells, please explain.
Cortical neurons were used for miRNA mimic experiments (Fig. 4c), since these cells have lower endogenous miRNA levels so that the mimic effect is more pronounced. Hippocampal neurons however were used for PTX experiments (Fig. 4d), since these cultures are more uniform and robustly engage in synaptic downscaling in response to 48h PTX treatment (Fiore et al., 2014).

- Data validating the level of expression for each mimic used is needed, particularly because an unrelated mimic (miR-495) was used to demonstrate miRNA specificity. Without this validation, one may speculate whether these effects are due to these specific miRNAs or if, for example, transfection with the miR-495 mimic was not as efficient.
We have now performed a relative quantification of miRNA levels by qPCR upon transfection of different miRNA mimics into cortical neurons (Appendix Figure S10). Thereby, we did not obtain any evidence that miR-495 transfection might be less efficient. If anything, it appears that the level of overexpression achieved for this miRNA is higher compared to miR-329 and miR-485.

- It is unclear what the controls used in all the assays were. Please define more directly.
In all experiments using miRNA mimics or anti-miRs, oligonucleotides with a sequence that does not target any cellular miRNA were used as controls. This has now been specified in more detail in the materials and methods section (p. 14).

Figure S8.
b. Assay results with the unrelated mimic must be shown for Cnih2 and Scr, as well.
Since we have already included a miRNA mimic with an unrelated sequence in the luciferase assays (Fig. 4c; “CTR” conditions), we do not feel that an additional control condition is needed for these experiments. We also would like to point out that miR-495 is not an unrelated miRNA, since miR-495 is part of the miR379-410 cluster and a miR-495 binding site actually partially overlaps with the miR-329 site within the Prr7 3’UTR (targetscan.org). This site however appears non-functional in neurons, since transfection of a miR-495 mimic did not inhibit the expression of a Prr7 3’UTR luciferase construct (Fig. EV4b).
Methods.
Luciferase assay. 4 data points are shown per condition in the results; however, methods indicate transfections were performed in triplicates. Do data points represent average of technical triplicates for four independent experiments? Please define.
Each of the four data point represents an independent experiment (each of which was performed in triplicate).

Statistical Analysis. Authors must indicate which posthoc test was used following 1Way ANOVA analyses.
For One-way ANOVA, no posthoc test was used since we are only dealing with two groups (genotype and sex).

2nd Editorial Decision 7 November 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. Original referee #1 was not able to look at the revised manuscript again, but going through your point-by-point response, I consider his/her points as adequately addressed.

As you will see, referee #2 now supports the publication of your manuscript in EMBO reports. However, referee #3 has some further concerns, in particular regarding the statistical analyses, we ask you to address in a final revised version of the manuscript and/or a detailed point-by-point response.

Further, I have these editorial requests:

- The title is currently slightly too long. Could you provide a more compact title? Please note the title should have not more than 100 characters (including spaces). Do we need the name of the microRNA cluster in the title? How about:
"A placental-mammal specific microRNA cluster acts as a natural brake for sociability in mice"

- We would like to publish the paper as Scientific Report. For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do that for your manuscript. I think it would help this section if sub-headings were introduced. Please do that. For more details please refer to our guide to authors:
http://embor.embopress.org/authorguide#manuscriptpreparation

- Please check that all the figures conform to our guidelines. For some of the writing is rather small. Also the scale bars and boxes in Fig. 2A/B will be difficult to see in the final online version (please use thicker lines). See here our guide for figure preparation:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

- Could you please combine the Figures and their legends in the Appendix (put the legends below the respective figures), and update the TOC. I think this would be easier to follow for our readers.

- Please format the references according to our reference style. See:
http://embor.embopress.org/authorguide#referencesformat

- Please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries (comments), we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

When submitting your revised manuscript, we will require:
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted single figure files in high resolution (for main figures and EV figures if they have been modified)
- the revised Appendix

In addition I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as visual synopsis on our website.

I look forward to seeing the final 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 #2:

I am satisfied with the revised manuscript and author rebuttal. I have no further comments.

Referee #3:

The manuscript by Lackinger et al. investigates the role of the miR379-410 microRNA cluster in the hippocampus in the regulation of social behaviours via homeostatic synaptic downscaling. The suggested role of this microRNA cluster in disease states of the brain entailing abnormalities in social behaviours certainly enforces the relevance of this study. That being said, the potential downstream applications of these data in therapeutic approaches to these disease states makes the careful examination of the function of this microRNA cluster all the more important. Accordingly, while the authors' responses to the initial concerns are appreciated, the additional data added still does not sufficiently support the molecular role of this microRNA cluster.

Figure 3: Given that the authors have argued that an Ago-CLIP experiment is beyond the scope of this current paper, the additional validation of their suggested targets must be particularly strong. While attempts were made via RT-qPCR and Western blot analysis to confirm these targets both at the RNA and protein levels, the data presented could be stronger (see Fig 4b, Fig EV5, Appendix Fig 11-12).

Fig 4b
The extremely high degree of variability in the qPCR data presented, particularly in the wt samples, makes the data rather interpretable. For example, in Cnih2, it is alarming that certain samples have no expression of the gene and others have a 2-fold increase. It leads the reader to wonder whether there may have been some technical issues when the assay was performed. Moreover, the overexpression in several of the genes seems to be driven by one or two ko samples, some of which appear to be statistical outliers. For example, Dlgap3 and Src both seem to contain statistical outliers in the ko group.

Fig EV5, Appendix Fig 11-12
The authors have not convincingly shown that increases of proposed target mRNAs correspond to functional changes at the protein level, which is imperative if the authors would like to discuss how this microRNA cluster corresponds to proposed effects in homeostatic synaptic downscaling and social behaviours. While the authors do not rely heavily on the Cnih2 data for their claims, it should still be noted that the quality of the Cnih2 blot, particularly blot 1, would make it challenging to quantify. More importantly, the only target gene presented as being significantly upregulated at the protein level, Prr7, is not actually significantly upregulated when the correct statistical analysis is performed (unpaired student's t test in Fig 11-12 versus 1 sample t test Fig EV5 (see also minor criticisms: statistical analysis)). While it is true that whole tissue analysis can make it challenging to detect minor increases in a particular cell type or subcellular region, the authors could use IF analysis to circumvent these issues, which they have even commented. This approach is certainly not outside the scope of this current paper.

Minor Criticisms.
Figure 3.
While there is some evidence to suggest expression of this cluster may be neuronal-specific, it is unclear why the authors still have not taken the appropriate steps to validate this claim in ex vivo tissue. This validation is of the utmost importance given the downstream discussion/interpretation of their RNA-seq data. In particular, the data begs of this careful analysis given that the most enriched GO term is MHC Class II Protein complex. While this enrichment may reflect an inflammatory response in microglia secondary to the direct effects in neurons, without the expression validation in ex vivo tissue, it is impossible to say for sure. There is an extremely low percentage of microglia in primary neuronal cultures making it impossible to conclude from the in vitro analysis provided whether or not Mirg may be expressed in microglia, for example. The additional cited evidence (Jimenez-Mateos et al., 2012 and Allen Brain Atlas) also fail to definitively show that the

microRNAs expressed in this cluster are not expressed in non-neuronal cell types in the hippocampus.

Figure S5.
Given that a Sholl analysis examining dendritic complexity in miR379-410 ko compared to wt hippocampal pyramidal neurons was already presented in Vally et al., Nat. Neurosci. 2015, it is unclear how the images presented in this figure add to the claim that neuronal health and dendritic complexity were not affected.

Statistical Analysis.
- If the authors were examining the effects of genotype and sex as they say, then a Two-way ANOVA should have been performed rather than a 1Way ANOVA given that genotype and sex qualify as two variables rather than as two groups.
- Additionally, a Two-way ANOVA should have been used for the analysis of the luciferase data given that there were again two variables (mimic, 3' UTR construct). If the authors would instead like to treat these as 4 conditions rather than two variables each with two conditions, than a 1Way ANOVA with a posthoc test must be used. In either instance, it is not appropriate to use an unpaired student's t test as indicated in the figure legend, particularly because a comparison was made between conditions within these two separate variables.
- A 1 sample t test is not a valid t test to use when comparing means between two conditions as was the case in the Western Blot analysis.

2nd Revision - authors' response 16 November 2018

Referee #3:

The manuscript by Lackinger et al. investigates the role of the miR379-410 microRNA cluster in the hippocampus in the regulation of social behaviours via homeostatic synaptic downscaling. The suggested role of this microRNA cluster in disease states of the brain entailing abnormalities in social behaviours certainly enforces the relevance of this study. That being said, the potential downstream applications of these data in therapeutic approaches to these disease states makes the careful examination of the function of this microRNA cluster all the more important. Accordingly, while the authors' responses to the initial concerns are appreciated, the additional data added still does not sufficiently support the molecular role of this microRNA cluster.

We appreciate that this referee acknowledges that our revised manuscript is improved. However, as outlined in more detail below, we disagree that the additional data provided does not sufficiently support the molecular role of miR379-410. First, we have provided highly sensitive single molecule FISH data that clearly demonstrates the preferential expression of the miR379-410 precursor Mirg in hippocampal neurons, as opposed to glial cells (Fig. 3). Second, we have investigated the expression of ten potential miR379-410 target mRNAs by qPCR, all of which were significantly upregulated in the wt compared to the miR379-410 ko hippocampus (Fig. 4b). Lastly, we obtained evidence for a significant upregulation of one of the strongest candidates, Prr7, at the protein level by Western blot (Fig. EV5).

Figure 3: Given that the authors have argued that an Ago-CLIP experiment is beyond the scope of this current paper, the additional validation of their suggested targets must be particularly strong. While attempts were made via RT-qPCR and Western blot analysis to confirm these targets both at the RNA and protein levels, the data presented could be stronger (see Fig 4b, Fig EV5, Appendix Fig 11-12).

As detailed below, we are confident that our new data from qPCR and Western blot analysis, together with the data from luciferase assays, makes a rather strong case for several of the investigated candidate miR379-410 targets, including Prr7, Cnih2, Dlgap3 and Src.

Fig 4b
The extremely high degree of variability in the qPCR data presented, particularly in the wt samples, makes the data rather interpretable. For example, in Cnih2, it is alarming that certain samples have

no expression of the gene and others have a 2-fold increase. It leads the reader to wonder whether there may have been some technical issues when the assay was performed. Moreover, the overexpression in several of the genes seems to be driven by one or two ko samples, some of which appear to be statistical outliers. For example, Dlgap3 and Src both seem to contain statistical outliers in the ko group.

We strongly disagree with the opinion of this referee that our presented qPCR is highly variable. All the tested 10 candidates show an average of 1.5 – 2-fold induction at the RNA level, a magnitude which is in the range usually observed for microRNA regulation. The observed variance of the data points is also in the range usually observed when looking at gene expression changes in the mouse hippocampus in vivo, as opposed to for example cell lines. Most importantly, appropriate statistical evaluation of the data demonstrated that for all the tested candidates, the differences between wt and ko datasets were statistically significant ($p>0.05$). Our statistical analysis also did not reveal any outliers that should have been removed.

Fig EV5, Appendix Fig 11-12

The authors have not convincingly shown that increases of proposed target mRNAs correspond to functional changes at the protein level, which is imperative if the authors would like to discuss how this microRNA cluster corresponds to proposed effects in homeostatic synaptic downscaling and social behaviours. While the authors do not rely heavily on the Cnih2 data for their claims, it should still be noted that the quality of the Cnih2 blot, particularly blot 1, would make it challenging to quantify. More importantly, the only target gene presented as being significantly upregulated at the protein level, Prr7, is not actually significantly upregulated when the correct statistical analysis is performed (unpaired student's t test in Fig 11-12 versus 1 sample t test Fig EV5 (see also minor criticisms: statistical analysis)). While it is true that whole tissue analysis can make it challenging to detect minor increases in a particular cell type or subcellular region, the authors could use IF analysis to circumvent these issues, which they have even commented. This approach is certainly not outside the scope of this current paper.

We appreciate that this referee admits that whole tissue analysis can make it challenging to detect minor increases in a particular cell type or subcellular regions. Accordingly, we observed about 1.2 – 1.3-fold increases in expression of two of the investigated targets, Prr7 and Cnih2, in the hippocampus of miR379-410 ko compared to wt mice. For the quantification of the Western blot data we had to analyze multiple blots due to the high number of samples analysed. We chose to use normalized data (the average of wt was set to 1; Fig. EV5A), since the absolute intensity of the Western blot bands can vary between different blots due to a variety of technical reasons (different blotting efficiencies, different degrees of antibody hybridization, etc.). Concerning statistical analysis, we are highly confident that the use of a one-sample t-test is appropriate in this case, since the normalization of the data naturally leads to a loss of variance in the dataset that was set to one (in this case the wt condition). Nevertheless, we also performed a statistical analysis on the un-normalized values, and the difference almost reached statistical significance ($p=0.055$) for Prr7. We would like to emphasize that these differences in P value are not due to a less stringent statistical analysis, but due to the effect of normalization that is routinely used to take into account the unavoidable technical variability of biological samples. Taken together, our Western blot data provides evidence for an upregulation of Prr7 protein levels in the miR379-410 ko compared to wt hippocampus, and a trend towards upregulation for Cnih2.

Furthermore, we agree with the referee that IF could support our findings from Western blot. However, we could not convince ourselves about the specificity of the commercially available antibodies for Prr7 with regard to IF up to this point, and a more stringent evaluation of these or newly generated antibodies would require Prr7 ko tissue, which is currently not available in our laboratory and therefore clearly beyond the scope of the present study.

Minor Criticisms.

Figure 3.

While there is some evidence to suggest expression of this cluster may be neuronal-specific, it is unclear why the authors still have not taken the appropriate steps to validate this claim in ex vivo tissue. This validation is of the utmost importance given the downstream discussion/interpretation of their RNA-seq data. In particular, the data begs of this careful analysis given that the most enriched GO term is MHC Class II Protein complex. While this enrichment may reflect an inflammatory response in microglia secondary to the direct effects in neurons, without the expression validation in ex vivo tissue, it is impossible to say for sure. There is an extremely low percentage of microglia in

primary neuronal cultures making it impossible to conclude from the in vitro analysis provided whether or not Mirg may be expressed in microglia, for example. The additional cited evidence (Jimenez-Mateos et al., 2012 and Allen Brain Atlas) also fail to definitively show that the microRNAs expressed in this cluster are not expressed in non-neuronal cell types in the hippocampus.

We agree with this reviewer that a number of lines of evidence suggest that the expression of the cluster in the brain is neuronal-specific, although spurious expression in non-neuronal cell types (e.g. microglia) cannot be formally ruled out.
If such an expression indeed exists, it however would not significantly affect the interpretation of our data. The RNA-seq data clearly demonstrates that loss of miR379-410 expression affects the expression of neuron-specific genes involved in ionotropic glutamate receptor function, and this specific gene expression profile correlates with cellular alterations in hippocampal neurons at the level of synaptic morphology and physiology. In the context of a constitutive knockout model, changes in other cell systems can hardly be ruled out. Thus, it is still plausible that they could contribute in a non-cell-autonomous manner to the observed phenotypes.
That said, since miRNA expression has to reach a certain cellular level to be physiologically meaningful (e.g. Bartel, Cell 2018), we consider a contribution of (micro)glia derived miR379-410 to the observed phenotypes highly unlikely. First, microRNA profiling studies could not detect significant expression of miR379-410 members in microglia (Varol et al., Immunity 2017; Hoye et al., J. Neurosci. 2017). Second, Landgraf et al., did not detect significant expression levels of miR379-410 members in cell lines of glial origin. Third, Jimenez-Mateos et al. (Nat. Med. 2012) found that expression of miR-134 is restricted to principal neurons within the hippocampus formation by in situ hybridization. Finally, our own highly sensitive single molecule FISH data for the miR379-410 precursor Mirg (Fig. 2b) in primary hippocampal neurons showed highly specific and exclusive staining of Mirg in the nuclei of MAP2-positive neurons. This definitely rules out expression of miR379-410 in astrocytes, which are abundant in our cultures, and possibly also in the low percentage of microglia usually present in primary hippocampal neuron cultures. Taken together, miR379-410 miRNAs are extremely lowly expressed, if not completely absent, from non-neuronal cell types in the rodent hippocampus.

Figure S5.
Given that a Sholl analysis examining dendritic complexity in miR379-410 ko compared to wt hippocampal pyramidal neurons was already presented in Vally et al., Nat. Neurosci. 2015, it is unclear how the images presented in this figure add to the claim that neuronal health and dendritic complexity were not affected.

We agree that the absence of a dendritic phenotype in miR379-410 deficient hippocampal neurons had already been demonstrated by us in a previous publication (Valluy et al., Nat. Neurosci. 2015). However, we would like to still include this data in the final manuscript, since it independently confirms these results in a slightly different mouse line that was used for multiphoton imaging (Thy1-GFP/miR379-410 ko).

Statistical Analysis.
- If the authors were examining the effects of genotype and sex as they say, then a Two-way ANOVA should have been performed rather than a 1Way ANOVA given that genotype and sex qualify as two variables rather than as two groups.

We fully agree with this comment. Maybe it escaped the attention of this reviewer, but we had already included a two-way ANOVA statistical analysis of the interaction between genotype and sex for the behavioural data in the Appendix file of the revised manuscript (**Appendix: ANOVAs with genotype and sex between-subject factors).** We have now also included this information in the Material and Methods section ("statistical analysis").

- Additionally, a Two-way ANOVA should have been used for the analysis of the luciferase data given that there were again two variables (mimic, 3' UTR construct). If the authors would instead like to treat these as 4 conditions rather than two variables each with two conditions, than a 1Way ANOVA with a posthoc test must be used. In either instance, it is not appropriate to use an unpaired

student's t test as indicated in the figure legend, particularly because a comparison was made between conditions within these two separate variables.

We appreciate this valuable suggestion, and have accordingly re-analyzed the luciferase data presented in Fig. 4 using two-way ANOVA followed by appropriate post-hoc testing for individual comparisons. We have now also included this information in the Material and Methods section ("statistical analysis").

- A 1 sample t test is not a valid t test to use when comparing means between two conditions as was the case in the Western Blot analysis.

We strongly disagree with this statement, since we were actually comparing **normalized** data whereby the control group was set to one. In this case, one-sample t-test is the most appropriate and stringent statistical test. Please see also our comments above regarding the analysis of the Western blot data.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Gerhard Schratt
Journal Submitted to: EMBOR
Manuscript Number: EMBOR-2018-46429V1 (revised)

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

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

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 used to pre-determine sample size but we orientated and tried to adapt our sample sizes to those used and reported in previous publications (e.g. Valluy et al., 2015; Fiore et al., 2014)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For animal studies no statistical methods were used to pre-determine sample size but we orientated and tried to adapt our sample sizes to those used and reported in previous publications (Wöhr et al., 2013; Sungur et al., 2016; Sungur et al., 2017).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Exclusion criteria and outliers are described in the method section of the manuscript.
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.	Electrophysiology data aquisition was performed by an investigator with knowledge of the identity of the experimental group. For behavioural tests, animals were given a code combination of letters and numbers to blind the animals for all tests. In addition, if not indicated explicitly in the methods section, behaviour experiments were controlled by computer systems to collect and analyze data in an automated and unbiased way.
For animal studies, include a statement about randomization even if no randomization was used.	All experiments performed in this study were randomized. Animals were assigned randomly in the various experimental groups and handling of animals were performed equally. We described randomization in the method section of the manuscript.
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.	Blinding and randomization were conducted when possible in the animal studies as mentiond below and in the method section.
4.b. For animal studies, include a statement about blinding even if no blinding was done	As mentioned above (point "3"), for behavioural tests, animals were given a code combination of letters and numbers to blind the animals for all tests. In addition, if not indicated explicitly in the method section, behaviour experiments were controlled by computer systems to collect and analyze data in an automated and unbiased way.
5. For every figure, are statistical tests justified as appropriate?	Yes, we performed appropriate statistical tests for every figure.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Single data points are shown in every figure to display variance and distribution. Appropirate tests are indicated in the figures and statistic is described in the method section.
Is there an estimate of variation within each group of data?	Regarding the behaviour data we performed statistics to investigate potential gender effect. Thereby, we did not detect any differences between the sexes except for the elevated plus maze (EPM) test (Fig. EV4d), where the genotype effect is mainly in females. A detailed statistical assessment of the behavioural data with respect to gender differences can be found in the Appendix file.
Is the variance similar between the groups that are being statistically compared?	Individual data points are displayed in the figures to show the variance.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	anti-Cnih2, species: polyclonal rabbit, source: Synaptic Systems, 253203, Lot 253203 / 3 anti-Prr7 (TRAP3 / 10), species: monoclonal mouse, source: Thermo Fisher Scientific, MA1-10448, Lot 74833187
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used. All experiments were performed in primary neuronal culture of mice (miR379-410 wt/ko) and rats (Sprague-Dawley rats) as described in the material section.

* 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.	C57Bl6/N6 mice (4-6 week-old and 3-month old adults) were used for three-chamber social memory test as stimulation subjects and are available at Charles River (Sulzfeld, Germnay). The conditional knockout mouse line miR379-410 (P0-P224, please see method section for more details), that were used for molecular, cellular and behavioural experiments were produced at Taconic Artemis (Cologne, Germany) and described previously in Valluy et al., 2015. The Thy1GFP reporter mice (3-month old) that were used for spine analysis were gifted by Marco Rust (Institute for Physiological Chemistry, University Marburg). Sprague-Dawley rats that were used for primary neuronal culture are available at Harlan-Winkelmann (Borchen, Germany). For reciprocal social interaction test, P22 juvenile mice were housed in isolation for 24h before testing. Otherwise, all rodents were housed under standard cage conditions with food and water ad libitum and maintained on a 12 h / 12 h light/dark cycle.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were performed in accordance with the animal protection law of Germany and were approved by the local authorities responsible for the Philipps University Marburg (Regierungspräsidium, Gießen, Germany).
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.	As indicated above, all animal experiments were performed in accordance with the animal protection law of Germany.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The RNAseq data is currently submitted to the Gene Expression Omnibus (GEO) database, submission no. PRJNA494281. Accession codes will be provided upon availability.
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).	N/A
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).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

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.	N/A