EMBO reports

Hippocampal CD39/ENTPD1 Promotes Mouse Depression-like Behavior through Hydrolyzing Extracellular ATP

Qian-Qian Cui, Zhuang-Li Hu, Yuan-Lang Hu, Xi Chen, Ji Wang, Li Mao, Xiao-Jia Lu, Ming Ni, Jian-Guo Chen, Fang Wang

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Transaction Report:

(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 4 March 2019

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We have now received the full set of referee comments that is pasted below.

As you will see, all referees acknowledge that the findings are potentially interesting. However, referees 2 and 3 also point out that significant revisions are required before the study can be considered for publication here. They note missing controls, the requirements for further tests and clarifications. Given that the comments are largely in agreement, I think that all of them should be addressed.

We would thus 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 6 main figures, I suggest that you layout the revised manuscript as a full article (i.e. more than 5 figures and separate results and discussion sections).

Supplementary figures, tables and movies can be provided as Expanded View (EV) files, and we can offer a maximum of 5 EV figures per manuscript (plus EV movies and tables). EV figures are embedded in the main manuscript text and expand when clicked in the html version. Additional supplementary figures will need to be included in an Appendix file. Tables can either be provided as

regular tables, as EV tables or as Datasets. Please see our guide to authors for more information.

Regarding data quantification, please specify the number "n" for how many independent 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 must be provided in the figure legends. Please also include scale bars in all microscopy images.

We 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.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where in the manuscript the requested information can be found. The completed author checklist will also be part of the RPF (see below).
- 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 figure files in high resolution. In order to avoid delays later in the process, please read our figure guidelines before preparing your manuscript figures at: http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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:

This is an interesting paper elucidating the mechanisms whereby a long-lasting decrease of ATP concentrations in the hippocampus are causally related to the development of depression in mice undergoing chronic stress.

I have only minor points of criticism to raise:
1. CD39 and ENTPD1 is used alternately. Would it not better to stick to one of the two terms.
2. In Fig. 1 the expression of the CD39 gene was shown to increase, while that of the ENPTD2 mRNA was reduced. Apparently the effect of CD39 pre-dominates. This could be mentioned already here.
3. In Fig. 2 it astonishes that 80 U/ml apyrase is effective, while the higher concentration is not. Any explanation?
4. In the Discussion it should be pointed out more decisively, that the authors suggest two unrelated

mechanisms for depression-like behavior in the hippocampus: (1) ATP-induced modulation of neural progenitor cell proliferation; (2) ATP-induced modulation of spine density on the pyramidal cells and molecular cells.
5. Some explanation for the divergent results observed in the mPFC (decreased ATP release) and hippocampus (more rapid degradation of ATP) should be given.
6. Please cite an article which suggests that AMP is a weak agonist on P2YRs.
7. Is the decrease in CD39 quantity/activity a reason or a consequence of the low ATP concentration in the hippocampus? How do the authors exclude that a lower release is the primary effect of chronic stress?

Referee #2:

This is an interesting study that addresses a hot issue in current neurological research. Purinergic signaling is increasingly thought to be important in the regulation of CNS physiology and in the determination of depressive behaviors. A member of the P2 receptor family (P2X7) is actively investigated as a potential target for the treatment of bi-modal disorders. The underlying assumption is that, especially in disease conditions, extracellular ATP reaches concentrations sufficient to activate this low-affinity receptor. Thus, in principle this study is interesting and timely. However, I have some concerns that should be addressed.

Major criticism

1. An key proof for the role plaid by extracellular ATP is the effect of apyrase. I warn the authors about the use of this ATP-hydrolyzing enzyme in the absence of proper controls (see PMID: 29382767). Proper controls should be run with inactivated (e.g. boiled) apyrase.

2. Now, genetically-engineered probes for the in vivo measurement of extracellular ATP are available (see PMID: 28683062). I understand that this might be technically demanding, but it will for sure substantially increase the quality of the paper.

3. It is not clear to me why ARL67156 reversed the social avoidance but did not restore sucrose preference (Fig. 3).

4. CD39-KO mice are available. I wonder why they are not even mentioned. Do they also have a depressive behavior?

5. Resilient animals were dismissed in this study, however it would be very interesting to know if resiliency was linked to extracellular ATP levels or CD39 expression.

Minor criticism

1. Introduction. ATP is also actively released by microglia. This should be mentioned.

Referee #3:

In the present manuscript, Hu et al. investigate the function of the extracellular ATPase CD39 in the regulation of Depressive-like behaviour in mice. By using a combination of behavioural, pharmacological and cell biological approaches, the authors identify CD39 as a positive regulator of depressive-like behaviour and a negative regulator of neurogenesis and spine growth in the hippocampus. In general, these are interesting findings that describe a novel pathway in the control of social and depressive-like behaviour in mice which could also represent a druggable target in the future. Unfortunately, many of the conclusions provided by the authors were not fully supported by the presented datasets. As outlined in more detail below, critical control conditions are missing, many of the experiments were performed in a rather superficial way and the actual meaning of the results is very often overstated. Thus, I would recommend that the authors carefully revise their manuscript according to my suggestions before it should be considered for publication in EMBO Reports.

General concerns:
1. in their title, the authors state that they identified a positive role of CD39 in depressive-like behaviour. However, this statement largely rests on a single test (sucrose preference) that in fact is used to assess "depressive-like" behaviour in rodents. The other test (social avoidance) is a rather generic indicator of emotional state. It is therefore imperative that the authors include at least one other test in this domain (e.g. forces swim) in the context of their CD39 manipulations.
2. Based on pharmacological and genetic manipulation of CD39, the authors state that CD39-dependent ATP hydrolysis is involved in the control of depressive-like behaviour. This is however a strong overstatement, since several of the treatments (Fig. 2H, 3E) in fact only rescued the social aspect of the phenotype, but not anhedonia (>sucrose preference test). One could even argue that these results suggest that the regulation of CD39 by CSDS in the hippocampus appears not to be involved in depression. Such an interpretation would also be supported by the observation that inhibition of CD39 increases neurogenesis in the dentate gyrus even in the absence of CSDS. In this regard, it is also puzzling why no data on sucrose preference is presented in the CD39 siRNA experiments (Fig. 5), which gave strong effects on most of the other parameters.
3. Concerning siRNA experiments, these are unfortunately missing a very critical control, namely an infection with a virus that expresses an unrelated siRNA. Multiple studies have reported potential non-specific effects due to engagement of the siRNA pathway, so such a control is absolutely critical. Even better would be the use of an shRNA-resistant CD39 to rescue the CD39 siRNA effects.

More specific concerns:
1. it would be desirable to perform the neurogenesis and dendritic spine readouts both in the context of the pharmacological and genetic manipulation of CD39.
2. the degree of CD39 knockdown (Fig. 5C) is very low. Is this due to the fact that most of the cells are not infected, or is the effect low in each of the infected cells (in which case it would be rather surprising to observe such strong phenotypes).
3. interestingly, the authors observe a rather specific effect on stubby spines (Fig. 6), but do not further comment on the potential relevance of this result.
4. the authors state that it is "unclear whether ARL specifically antagonized CD39 in vivo". Given this uncertainty, they should provide some evidence that at least CD39 is inhibited with this drug (similar to Fig. EV4, where ATPase activity was measured).
5. CSDS is also known to affect anxiety-like behaviours, so additional tests in this domain should be included (e.g. EPM, open field)
6. in general, the manuscript is rather poorly written, with many typos, missing page and figure numbers, etc.

1st Revision - authors' response 23 July 2019

Response to the reviewers' comments:

Referee #1:

This is an interesting paper elucidating the mechanisms whereby a long-lasting decrease of ATP concentrations in the hippocampus are causally related to the development of depression in mice undergoing chronic stress.
I have only minor points of criticism to raise:
1. CD39 and ENTPD1 is used alternately. Would it not better to stick to one of the two terms.
Response*: We would like to thank the reviewer for the positive comments and good suggestion. In the revision, we have unified CD39 and ENTPD1 as CD39.*

2. In Fig. 1 the expression of the CD39 gene was shown to increase, while that of the ENPTD2 mRNA was reduced. Apparently, the effect of CD39 pre-dominates. This could be mentioned already here.
Response*: We agree with the reviewer' comments. As suggested, we have added this conclusion in the revised manuscript (see Page 6, Line 7-8).*

3. In Fig. 2 it astonishes that 80 U/ml apyrase is effective, while the higher concentration is not. Any explanation?
Response*: We thank the reviewer for pointing out this issue. For the result that CD39 analog apyrase in 80U/ml induced depression-like behaviors, while the higher concentration was not*

effective, we thought it may be due to the two determinants of extracellular ATP level, ATPase hydrolyzing and direct cellular releasing [1]. When there is a much higher concentration apyrase to hydrolyze ATP, it may trigger cell releasing to compensate for the ATP loss [2]. Thus, the releasing ATP will prevent the depression-like behaviors. In the revision, we have added the explanation in discussion section (see Page 14, Last line 1-2 to Page 15, Line 1-5).

4. In the Discussion it should be pointed out more decisively, that the authors suggest two unrelated mechanisms for depression-like behavior in the hippocampus: (1) ATP-induced modulation of neural progenitor cell proliferation; (2) ATP-induced modulation of spine density on the pyramidal cells and molecular cells.
Response*: We thank the reviewer for the constructive suggestion. There are indeed two mechanisms for CD39 induced depression-like behavior in the hippocampus. In the revised manuscript, we clarified the contributions of neural progenitor cell proliferation and spine density to CD39 induced depression-like behavior in the discussion section (see Page 17, 2nd paragraph, Line 2-5).*

5. Some explanation for the divergent results observed in the mPFC (decreased ATP release) and hippocampus (more rapid degradation of ATP) should be given.
Response: *Thanks for your suggestion. In our present study, we found that CD39 expression and activity increased in the hippocampus (more rapid degradation of ATP) of defeated mice, but not in the mPFC, while others found deficiencies in astrocytic ATP release from mPFC and hippocampus causing depressive-like behaviors [3]. The divergent results between the mPFC and hippocampus may be due to the different brain structure and cellular component, and the changes of ATP induced by stress have brain region specificity. We have given the explanation in the discussion section (see Page 14, Line 10-14).*

6. Please cite an article which suggests that AMP is a weak agonist on P2YRs.
Response*: We apologize for the missing citation. In the revised manuscript, we have cited the reference "28" on Page 16, Line 1.*

7. Is the decrease in CD39 quantity/activity a reason or a consequence of the low ATP concentration in the hippocampus? How do the authors exclude that a lower release is the primary effect of chronic stress?
Response*: Thank you for raising an important question. CD39 is a major hydrolase for extracellular ATP and hydrolyzes ATP and ADP into AMP [4], so we predict that the increase in CD39 quantity results in the low ATP concentration. In addition, in our present study, genetic deletion of CD39 restored the declined level of ATP in the hippocampus of mice subjected to 10-d social defeat stress (Fig. 4H), and ATP infusion could reverse social avoidance that induced by apyrase exposure (Fig. 2G). These results support that the increase in CD39 quantity is a reason of the low ATP concentration in the hippocampus and is the primary effect of chronic stress. However, it has been reported that stimulating endogenous ATP release from astrocytes induced antidepressant-like effects in mouse models of depression [3], so we could not exclude the role of a lower ATP release in chronic stress. We have mentioned these issues in the discussion section (see Page 15, Line 8-13).*

Referee #2:

This is an interesting study that addresses a hot issue in current neurological research. Purinergic signaling is increasingly thought to be important in the regulation of CNS physiology and in the determination of depressive behaviors. A member of the P2 receptor family (P2X7) is actively investigated as a potential target for the treatment of bi-modal disorders. The underlying assumption is that, especially in disease conditions, extracellular ATP reaches concentrations sufficient to activate this low-affinity receptor. Thus, in principle this study is interesting and timely. However, I have some concerns that should be addressed.
Major criticism
1. An key proof for the role plaid by extracellular ATP is the effect of apyrase. I warn the authors about the use of this ATP-hydrolyzing enzyme in the absence of proper controls (see PMID: 29382767). Proper controls should be run with inactivated (e.g. boiled) apyrase.
Response: *We thank the reviewer for pointing out this issue. According to the suggestion, we added the control with boiled apyrase and found that it had no effect on depressive behaviors of mice (Fig. EV2B-E). So the effect of ATP is based on the ATP-hydrolyzing enzyme, and increased CD39 activity in the hippocampus contributes to the depression-like behaviors. We have described the results in the revised manuscript (see Page 7, Line 14-20).*

2. Now, genetically-engineered probes for the in vivo measurement of extracellular ATP are available (see PMID: 28683062). I understand that this might be technically demanding, but it will for sure substantially increase the quality of the paper.

Response: *Thanks for the good suggestion and we agree that genetically-engineered probes for the in vivo measurement of extracellular ATP would be much better. However, it is a little difficult for us to establish a new technology within a short time, so we chose a bioluminescent ATP assay kit (Promega, Leiden, The Netherlands) to examine the extracellular ATP level in hippocampal slice [3, 5-7]. We are so sorry for the absence of probes application, and we will try to carry out in vivo measurement in subsequent research.*

3. It is not clear to me why ARL67156 reversed the social avoidance but did not restore sucrose preference (Fig. 3).
Response: *Thank you for pointing out the question. In our study, CD39 inhibitor ARL67156 only reversed the social avoidance but did not restore sucrose preference when infused it to hippocampus (Fig 3E and F), however, both behaviors could be reversed by infusion of ARL67156 to the right cerebral ventricle (Fig 3C and D). Thus, we speculated that other brain regions besides hippocampus might be response to the antidepressant effects of CD39 inhibitor (description in Page 13, Last line 1-3 to Page 14, Line 1-2). However, in order to confirm the antidepressant effects of hippocampal ARL67156, we performed additional desperate behavior experiments, TST and FST (Fig. 3G and H). The improvement of depressive behaviors induced by hippocampal ARL67156 administration verified the antidepressant-like effects of hippocampal CD39 inhibition. We described the result in the revision (Page 9, Line 8-9).*

4. CD39-KO mice are available. I wonder why they are not even mentioned. Do they also have a depressive behavior?
Response: *Sorry for not using the CD39-KO mic due to lack of a colony of CD39-KO mice. It has been reported that CD39$^{-/-}$ mice have reduced levels of anxiety and the cerebrospinal fluid of CD39$^{-/-}$ mice contain increased levels of ATP compared with WT mice [8]. Cao et al. also found that mice with low ATP in the brain were susceptible to chronic social defeat stress and administration of ATP had antidepressant-like effect [3]. In addition, studies have shown that ATP promotes adult neural stem cell proliferation, rescues hippocampal neurogenesis [9] and prevents Aβ_{42}-mediated dendritic spine loss [10], which are assistant with our results. So we speculate that CD39$^{-/-}$ mice might have antidepressant actions. However, in order to confirm the role of CD39 in depression, pharmacological inhibition and genetic silencing techniques were adopted, both treatment uniformly produced an antidepressant-like effect (Fig 3 and 4). Moreover, when we use LV-siCD39, we can inject viral vectors into hippocampus to target on hippocampal CD39. It can be inferred from our results that CD39 knockout mice may have an antidepressant effect, which needs our further research.*

5. Resilient animals were dismissed in this study, however it would be very interesting to know if resiliency was linked to extracellular ATP levels or CD39 expression.
Response: *We thank the reviewer for the good suggestion and agree that resilient animals should be investigated here. In the revised manuscript, we detected the relationship between resilient animals and extracellular ATP levels or CD39 expression (Fig. EV1A-C). The results showed that the extracellular ATP levels and CD39 expression were unchanged in resilient mice. So, the increase in the expression and activity of CD39 were only occurred in the hippocampus of susceptible mice. We have described the result in the revised version (Page 6, Line 8-11).*

Minor criticism
1. Introduction. ATP is also actively released by microglia. This should be mentioned.
Response: *Thank you for your kind remind. We have added the description "In addition, a large amount of ATP can be released from microglia, particularly during neuroinflammation" in the introduction section. (Page 3, 2nd paragraph, Line 2-3).*

Referee #3:

In the present manuscript, Hu et al. investigate the function of the extracellular ATPase CD39 in the regulation of Depressive-like behaviour in mice. By using a combination of behavioural, pharmacological and cell biological approaches, the authors identify CD39 as a positive regulator of depressive-like behaviour and a negative regulator of neurogenesis and spine growth in the hippocampus. In general, these are interesting findings that describe a novel pathway in the control of social and depressive-like behaviour in mice which could also represent a drugable target in the future. Unfortunately, many of the conclusions provided by the authors were not fully supported by the presented datasets. As outlined in more detail below, critical control conditions are missing, many of the experiments were performed in a rather superficial way and the actual meaning of the results is very often overstated. Thus, I would recommend that the authors carefully revise their manuscript according to my suggestions before it should be considered for publication in EMBO Reports.

General concerns:

1. in their title, the authors state that they identified a positive role of CD39 in depressive-like behaviour. However, this statement largely rests on a single test (sucrose preference) that in fact is used to assess "depressive-like" behaviour in rodents. The other test (social avoidance) is a rather generic indicator of emotional state. It is therefore imperative that the authors include at least one other test in this domain (e.g. forces swim) in the context of their CD39 manipulations.
Response: *We thank the reviewer for the constructive suggestion. In the revised manuscript, we added desperate behavior experiments, e.g., forces swim test (FST) and tail suspension test (TST) to assess depressive-like behaviors (Fig. 1D and E, described in Page 5, Line 10-12) and the role of CD39. Similar to the result in social interaction test, pharmacological inhibition (Fig. 3G and H, described in Page 9, Line 8-9) and genetic silencing (Fig. 4E and F, described in Page 10, Line 1-2) of CD39 both significantly reduce the immobility time of susceptible mice in FST and TST. Thus, the uniform results indicate a positive role of CD39 in depressive-like behavior.*

2. Based on pharmacological and genetic manipulation of CD39, the authors state that CD39-dependent ATP hydrolysis is involved in the control of depressive-like behaviour. This is however a strong overstatement, since several of the treatments (Fig. 2H, 3E) in fact only rescued the social aspect of the phenotype, but not anhedonia (>sucrose preference test). One could even argue that these results suggest that the regulation of CD39 by CSDS in the hippocampus appears not to be involved in depression. Such an interpretation would also be supported by the observation that inhibition of CD39 increases neurogenesis in the dentate gyrus even in the absence of CSDS. In this regard, it is also puzzling why no data on sucrose preference is presented in the CD39 siRNA experiments (Fig. 5), which gave strong effects on most of the other parameters.
Response: *Thanks for raising the issue about sucrose preference test. In our study, sucrose preference is absent in the CD39 siRNA experiments, this is because anhedonia cannot persist for 4 weeks in CSDS mice [11], however, it will take more than 3 weeks to achieve the best knockdown effect on CD39 with LV-siCD39, so it is difficult to evaluate the effect of LV-siCD39 on anhedonia. Therefore, in order to assess the role of CD39 in depressive-like behaviors, we added desperate behavior experiments, forces swim test (FST) and tail suspension test (TST) in the revised manuscript. The significant reduction in the immobility time of susceptible mice after pharmacological inhibition (Fig. 3G and H, described in Page 9, Line 8-9) and genetic silencing (Fig. 4E and F, described in Page 10, Line 1-2) of CD39, together with the alleviation in social avoidance in SI test sufficiently indicate that CD39 mediates depressive-like behavior.*
As for the inhibition of CD39 increases neurogenesis in the dentate gyrus even in the absence of CSDS, we thought it may be related to the increase in the extracellular ATP after inhibition of CD39, and then high level of ATP induces neurogenesis [9]. The regulation of neurogenesis by CD39 should be involved in depression, since both inhibition and knockdown of CD39 promoted hippocampal neurogenesis in susceptible mice (Fig 5 and corresponding results in Page 11, Last 2 lines to Page 12, Line 1-7.

3. Concerning siRNA experiments, these are unfortunately missing a very critical control, namely an infection with a virus that expresses an unrelated siRNA. Multiple studies have reported potential non-specific effects due to engagement of the siRNA pathway, so such a control is absolutely critical. Even better would be the use of an shRNA-resistant CD39 to rescue the CD39 siRNA effects.
Response: *We apologize for the unclear explanation. Here, we used lentiviral vector expressing GFP alone (LV-GFP) as a control. Actually□the LV-GFP control we used in the experiment was designed with an nonfunctional segment (Page 26, 2nd paragraph, Line 3-4). As for the determination of CD39 siRNA effects, we used recombinant mouse CD39 protein (CD39Fc) to replace the shRNA-resistant CD39, which had the same effect as shRNA-resistant CD39. The results showed that CD39Fc cancelled the antidepressant effects induced by LV-CD39-siRNA (Fig. EV5). The rescue effect of CD39Fc indicates that CD39 mediates the depression-like effects of mice (Page 10, 2nd paragraph).*

More specific concerns:
1. it would be desirable to perform the neurogenesis and dendritic spine readouts both in the context of the pharmacological and genetic manipulation of CD39.
Response: *Thanks for the suggestion. We have complemented the neurogenesis and dendritic spine readouts with pharmacological and genetic manipulation of CD39 in the revision. The results showed that knockdown of CD39 in the hippocampus increased the DG neurogenesis (Fig. 5F-H), consistently with the results treated with ARL67156. As well, CSDS induced decrease in density of stubby spine could be rescued by ARL67156 (Fig. 6F-J). We added the corresponding description in the result section (Page 12, Line 2-6; Page 12, Last 4 lines to Page 13, Line 1-5).*

2. the degree of CD39 knockdown (Fig. 4C) is very low. Is this due to the fact that most of the cells are not infected, or is the effect low in each of the infected cells (in which case it would be rather surprising to observe such strong phenotypes).
Response: *Thanks for your comments. Since the degree of CD39 knockdown was analyzed from 3 mice before, we repeated the infected experiments and increased the mice number to 6 in the revised*

version. The statistical result showed that the degree of CD39 knockdown is □50% (Fig. 4C). Sorry for our imprecision and we have rectified the value (Page 9, Line 19).

3. interestingly, the authors observe a rather specific effect on stubby spines (Fig. 6), but do not further comment on the potential relevance of this result.
Response: Sorry for our cursoriness. We have added the comment about the potential relevance of specific effect on stubby spines in the discussion section in the revision. (Page 16, Last line 1-7; Page 17, line 2-13).

4. the authors state that it is "unclear whether ARL specifically antagonized CD39 in vivo". Given this uncertainty, they should provide some evidence that at least CD39 is inhibited with this drug (similar to Fig. EV4, where ATPase activity was measured).
Response: *Thanks for the suggestion. According to the reviewer comment, we detected the effect of ARL67156 on ATPase activity. As predicted, the ATPase activity in hippocampus of susceptible mice was inhibited by ARL67156 (Fig. 3B, described in Page 8, Line 14-17).*

5. CSDS is also known to affect anxiety-like behaviours, so additional tests in this domain should be included (e.g. EPM, open field)
Response: *We agree with the reviewer that the anxiety-like behaviors should be included. In the revised manuscript, we added elevated plus maze (EPM) and open field test (OFT) to assess the effect of CD39 on anxiety-like behaviors. First of all, we found that CSDS induced anxiety-like behaviors of mice (Fig. 1F-H, Page 5, Line 12-14). Then, neither pharmacological inhibition (Fig. EV3C-E, Page 9, Line 11-12) nor genetic silencing (Fig. EV4E-G, Page 10, Line 4) of CD39 affected anxiety-like behaviors of mice.*

6. in general, the manuscript is rather poorly written, with many typos, missing page and figure numbers, etc.
Response: *We are so sorry for the poor written and mistakes. In the revised manuscript, we have checked the spelling, marked the pages and figure numbers. We hope the manuscript has been improved in writing and format.*

References

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2nd Editorial Decision 26 August 2019

Thank you for the submission of your revised manuscript. We have now received the full set of referee comments that is pasted below.

As you will see, while both referees 1 and 2 are satisfied with the revisions, referee 3 points out some more concerns that would need to be addressed before we can proceed with the handling of your manuscript here. If you decide to address these concerns, please also send us a point by point response.

I am looking forward to receiving a newly revised manuscript as soon as possible. Please let me know if you have any questions or comments.

REFEREE REPORTS

Referee #1:

My criticism was fully answered

Referee #2:

No more issues.

Referee #3:

The revised manuscript provided by Hu et al. is significantly improved. However, not all of my concerns have been adequately addressed:

1. The nature of the control construct "LV-GFP" used for the CD39 siRNA experiments is still mysterious. The authors mention that it contains a non-functional segment, but do not provide any further details. Does the construct contain a hairpin that gives rise to a non-functional siRNA? If so, what is the sequence of the non-functional siRNA?

2. Additional tests (TST, FST) in the depression domain have been included in the manuscript, and the results from these tests support the conclusion about a depression-promoting function of CD39. However, these tests are strongly underpowered (n=3 for some conditions!) and therefore the results can only be considered preliminary. Due to the high variability observed in mouse behavioural experiments, researchers usually consider n=10 sufficient, so additional animals have to be included.

3. The rescue experiment provided in Fig. EV5 unfortunately is missing important conditions that are required to draw any conclusions. In particular, conditions with the control siRNA are needed to judge whether CD39 knockdown gave the expected anti-depressive effect in this set of experiments and to also estimate the degree of rescue obtained with the recombinant CD39. Also, it would be important to measure the degree of CD39 overexpression achieved with this approach. Ideally, one would also like to see a non-related protein that does not rescue the phenotype to rule out non-specific effects.

4. The data presentation is still insufficient. Nowadays, it is pretty standard to provide the individual data points, ideally in the form of box plots. Also, the manuscript would benefit from a careful proofreading.

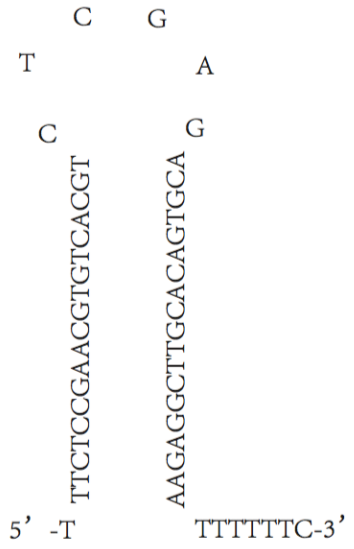
2nd Revision - authors' response 13 November 2019

Response to Reviewer #3

The revised manuscript provided by Hu et al. is significantly improved. However, not all of my concerns have been adequately addressed:
1. The nature of the control construct "LV-GFP" used for the CD39 siRNA experiments is still mysterious. The authors mention that it contains a non-functional segment, but do not provide any

further details. Does the construct contain a hairpin that gives rise to a non-functional siRNA? If so, what is the sequence of the non-functional siRNA?

Response: **We are very sorry for the confused explanation. The non-functional siRNA contains a hairpin, we have added the sequence of control siRNA: TTCTCCGAACGTGTCACGT in the revised manuscript (see Page 26, line 17-18). See the detail sequence as following:**



2. Additional tests (TST, FST) in the depression domain have been included in the manuscript, and the results from these tests support the conclusion about a depression-promoting function of CD39. However, these tests are strongly underpowered (n=3 for some conditions!) and therefore the results can only be considered preliminary. Due to the high variability observed in mouse behavioural experiments, researchers usually consider n=10 sufficient, so additional animals have to be included.

Response: **We are sorry for the insufficient animal number. We have added additional animals in TST and FST tests (see Page 38, line 15-17; Page 39, line 14-16, 18-20; Fig. 3G-H and Fig. 4E-F). Accordingly, we added other experimental data to a sufficient number in the revised manuscript (see Page 42, line 6-12 & Fig. 6G-J. Expanded View Page 3, line 5, 6, 9-12; Page 4, line 4, 7-12; Fig. EV3C-E, Fig. EV4C, EV4E-G).**

3. The rescue experiment provided in Fig. EV5 unfortunately is missing important conditions that are required to draw any conclusions. In particular, conditions with the control siRNA are needed to judge whether CD39 knockdown gave the expected anti-depressive effect in this set of experiments and to also estimate the degree of rescue obtained with the recombinant CD39. Also, it would be important to measure the degree of CD39 overexpression achieved with this approach. Ideally, one would also like to see a non-related protein that does not rescue the phenotype to rule out non-specific effects.

Response: **Thank you for your professional comments. We followed your suggestion and adjusted the experimental groups: Ctrl-LV-GFP-IgG, CSDS-LV-GFP-IgG, CSDS-LV-siCD39-IgG and CSDS-LV-siCD39-CD39Fc. The results showed that compared with CSDS-LV-GFP-IgG, CSDS-LV-siCD39-IgG could improve the depressive-like behaviors, and infusion of CD39Fc into hippocampus counteracted the effect of CD39 knockdown (Fig. EV5C-F). In addition, we used a non-related protein IgG, and IgG could not rescue the antidepressant action of siCD39 (Fig. EV5C-F). Also, we measured the degree of CD39 with CD39Fc infusion, and found that CD39Fc had about 50% overexpressed efficacy (Fig. EV5B) (see Page 10, line 12-14).**

4. The data presentation is still insufficient. Nowadays, it is pretty standard to provide the individual data points, ideally in the form of box plots. Also, the manuscript would benefit from a careful proofreading.

Response: **Thank you for your good suggestions. In the revised manuscript, we provided all data points in the form of box plots according to your suggestion.**

3rd Editorial Decision 6 December 2019

I sent you the email below about a month ago and hope that you have received it. I would like to accept your manuscript as soon as possible and given the very minor changes required, I was hoping to receive the final manuscript within a few days. Can you please let me know when you will be able to submit the final manuscript? Thank you.

Thank you for the submission of your revised manuscript. We have now received the comments from referee 3 and I am glad to tell you that we can in principle accept your paper now.

Only a few more minor changes will be required:

- Please address referee 3's comments in the final manuscript text

- Please ZIP all EV and Appendix source data into one single file. Please label all source data so that it is clear to which figure they refer

I also would like to suggest a few changes to the title and abstract (that needs to be written in present tense). Please let me know if you agree with the following:

Hippocampal CD39/ENTPD1 Promotes mouse Depression-like Behavior through Hydrolyzing Extracellular ATP

Emerging evidence indicates that low levels of ATP in the extracellular space may contribute to the pathophysiology of major depressive disorder (MDD). The concentration of extracellular ATP is regulated by its hydrolase ectonucleotide tri(di)phosphohydrolase (ENTPD). However, the role of ENTPD in depression remains poorly understood. Here we examine the role of CD39 (known as ENTPD1) in mouse depression-like behavior induced by chronic social defeat stress (CSDS). We demonstrate that CSDS enhances the expression and activity of CD39 in the hippocampus. The CD39 functional analog apyrase also induces depression-like behavior, which can be ameliorated by ATP replenishment. Pharmacological inhibition and genetic silencing of CD39 has an antidepressant-like effect via increasing hippocampal extracellular ATP concentration, accompanied with an increase in hippocampal neurogenesis and dendritic spine numbers in defeated mice. These results suggest that hippocampal CD39 contributes to CSDS-induced depression-like behavior via hydrolyzing extracellular ATP, indicating that CD39 may be a promising new target for the treatment of depression.

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

REFEREE REPORT

Referee #3:

After another round of revision, the manuscript was further improved and most of my previous concerns were adequately addressed. One remaining issue is that some of the conclusions made by the authors are not reflected by the actual results. For example, in new Fig. 3G-H, there is no significant (G) or only a marginal effect (H) of CSDS on the behaviour in the first place, and the CD39 inhibitor reduces immobility time irrespective of CSDS (in contrast to results from Fig. 3C-E). Similarly, CSDS does not have a significant effect on FST in Fig. 4F. The authors should at least discuss potential reasons for the observed discrepancies between tests. With these changes, the paper is ready for publication in EMBO Reports.

3rd Revision - authors' response 22 January 2020

Response to the reviewer #3

After another round of revision, the manuscript was further improved and most of my previous concerns were adequately addressed. One remaining issue is that some of the conclusions made by the authors are not reflected by the actual results. For example, in new Fig. 3G-H, there is no significant (G) or only a marginal effect (H) of CSDS on the behaviour in the first place, and the CD39 inhibitor reduces immobility time irrespective of CSDS (in contrast to results from Fig. 3C-E). Similarly, CSDS does not have a significant effect on FST in Fig. 4F. The authors should at least discuss potential reasons for the observed discrepancies between tests. With these changes, the paper is ready for publication in EMBO Reports.

Response: **Thank you for your positive comments on our revised work. In Fig. 3G-H and Fig. 4F, CSDS did not have a significant or only a marginal effect on TST and FST, which may result from the large dispersion among groups. Therefore, we repeated TST and FST to increase the animal numbers, and the results showed that there were significant effects of CSDS on despair behaviors of mice (Fig. 3G-H and Fig. 4E-F). In addition, CD39 inhibitor ARL67156 reduced immobility time irrespective of CSDS in Fig. 3G-H, suggesting that ARL67156 might have an inhibitory effect on acute stress (see Page 9, line 10-12; Page 10, line 4-5; Page 40, line 15-21; Page 41, line 16-22).**

Accepted 27 January 2020

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.

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: Jian-guo Chen and Fang Wang
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOR-2019-47857V1

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?	Different experiments have different size standard: in our manusript, behaviral tests need more sample size. Each experiments should be repeated more than 3 times. Western blotting and qPCR need about 4-8 size. Immunofluorescence need more than 3 size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Please see the description as 1.a.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We exclude the sample or animals by the behaviral results. Becaus the sample we used come from brain of C57BL/6J, we would exclude the data If the behaviral results of the animial abnormal.
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 animals' experiments we performed were randomly assigned to different groups.
For animal studies, include a statement about randomization even if no randomization was used.	Please see the description as 3.
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.	Yes, the reconstruction of 3D dendritic spines and analyse were performed by different persons.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Please see the description as 3.
5. For every figure, are statistical tests justified as appropriate?	Yes, all statistical tests are shown in figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Page on 26 in our manusript: Data are expressed as mean ± SEM and analyzed by employing the Graphpad 6.0 software. The unpaired student's t tests were used to compare differences between any given two groups throughout the study, unless otherwise specified. One-way ANOVA followed by Fisher's LSD test was used in different treatment groups where appropriate. Significance of multiple comparisons involving more than one variable was tested using two-way ANOVA followed by Tukey's post hoc test. A probability level of $P < 0.05$ was considered as statistically significant.
Is there an estimate of variation within each group of data?	Yes

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

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

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

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

Is the variance similar between the groups that are being statistically compared?	Yes, the coefficient of variation has been calculated for each sample.

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).	cd39: abcam (ab108248); DCX: Cell Signaling Technology(4604); BrdU: abcam (ab6326)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	C57BL/6J mice were purchased from Hunan SJA Laboratory Animal, Changsha, Hunan, China.8-month-old retired CD-1 breeders bought from Beijing Vital River Laboratory Animal Technology Co., Ltd. Beijing, China.They were group housed and maintained on a 12 h light/dark cycle with ad libitum access to food and water
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The research was conducted in accordance with the Guide for Care and Use of Laboratory Animals that adopted and promulgated by the National Institutes of Health. All experimental protocols were approved by the animal Welfare Committee of Huazhong University of Science and Technology
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.	We confirm this study complies to ARRIVE guidelines

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

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