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Postnatal expression of CD38 in astrocytes regulates synapse formation and adult social memory

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary

In their work submitted for review, Hattori et al. identify an astrocyte enriched protein (CD38) as important for social memory tasks in mice. The authors developed a conditional KO model to remove CD38 specifically in astrocytes using the GLAST-CreERT2 line crossed to a CD38 floxed line. The investigators use a three-chamber social approach test to show that loss of CD38 leads to reduced interaction time with a novel social stimulus only when the animal is given a break between test periods. The authors test whether changes in neuronal morphology or synapses in the medial prefrontal cortex (mPFC), a region important for social memory, can account for their behavioral phenotype. The researchers found that mPFC neurons in their conditional CD38 KO (cKO) animals have significantly less mature spines than wild-type (WT) controls. The authors then claim that this reduction in mature spines correlates with a reduction in VGluT1 positive excitatory synapse density in mPFC of cKO vs. WT. Next, the investigators use mass spectrometry of astrocyte conditioned media and neuronal cultures treated with astrocyte conditioned media to test whether a known astrocyte secreted synaptogenic factor, Sparcl1/Hevin, could underlie their reported changes in synapse density in their cKO animals. Finally, the authors use pharmacological inhibitors against different components of the CD38 signaling pathway to test whether CD38 regulates Hevin secretion by astrocytes. While the reported behavioral phenotype is interesting, this reviewer has several major concerns with the data claiming that reduction in Sparcl1/Hevin is underlying synaptic phenotypes in the CD38 cKO. Therefore, the paper is not suitable for publication without addressing the concerns listed below.

Major Concerns:

Synapse analysis in vivo: For the analysis of VGluT1 excitatory synapses in mPFC, it is not clear how the statistical analysis was performed. From the plotted error bars, it seems that the investigators used individual z-projections as the n for a t-test. This is inappropriate for this analysis as it would overinflate the N and down the p-value. It would be more appropriate to plot and compare animal averages between conditions or use a test that can account for the fact that there are repeated measures taken from the same animal. Additionally, the authors note a decrease in VGlut1+ puncta in the

global CD38 KO but no change in the protein levels in both the global and cKO.

Synapse analysis in vitro: The authors are missing key experimental controls for their analysis of synapse induction by astrocyte conditioned media. Firstly, the authors do not include a condition of neurons cultured alone without astrocytes or astrocyte conditioned media treatments. This is critical to this experiment because, without this control, it is impossible to assess the effectiveness of the astrocyte conditioned media or any recombinant protein treatments on synapse formation. Secondly, the authors give very few details and no supporting data about the purity of their neuronal cultures. This is critical to this experiment because any contaminating astrocytes in their cultures could severely skew the data for any given condition. Finally, the authors do not specify how they determined the doses for astrocyte conditioned media and Hevin treatments. The researchers give no details on how the astrocyte conditioned media was collected or treated before adding onto neurons. For this experiment to be viable, the researchers must collect the conditioned media in serum-free media, determine the protein concentration of their samples, and the dose-response to the astrocyte conditioned media must be performed to determine the optimal dose for each batch. When comparing between genotypes, this type of quality control is critical to assess whether there is, in fact, a difference in their synaptogenic capacity.

Western blots: All western blot quantification of astrocyte conditioned media should include total protein normalization. The authors do not describe how they normalize the astrocyte conditioned media blots, but without a total protein stain to normalize, it is impossible to be sure the same amount of protein was loaded into the gel for each lane. In Figure 3L, the western blot data showing the expression of VGluT1 and PSD95 should be improved, and a better representation is recommended. It is also strange that the CD38 cKO has no expression because CD38 is also expressed in endothelial cells. Why not isolate astrocytes from CD38 KO? Also, for VGluT1 and PSD95 western blots, it would be better to test mPFC lysates rather than whole cortical lysates.

Astrocyte morphogenesis: Since the astrocyte-specific deletion of CD38 from P10 impairs postnatal development of astrocytes, the authors should investigate if the impaired synaptogenesis seen in later stages is due to impaired astrocyte morphogenesis or the defect in the secretion of synaptogenic proteins like Sparcl1/Hevin or thrombospondins.

Mass spectrometry: There is no information about how many samples were used for mass spectrometry. This reviewer is concerned that this experiment may be underpowered given that other published datasets have identified significantly more proteins in wild-type ACM (about double than what was identified here). There needs

to be a quality assessment of the ACM to help ensure the production protocol can capture the full extent of proteins secreted by cultured astrocytes.

RNA sequencing: RNA sequencing results seem underpowered, and an accurate description of their collection methods is missing. It also seems to this reviewer that any prolonged culturing of the astrocytes would lead to additional transcriptional changes independent of their genetic manipulation. To avoid confounds due to culture artifacts, it might be cleaner to FACS sort astrocytes using a fluorescent reporter such as the Aldh1l1-eGFP line or RTM in their GLAST-creERT2 model. In the latter case, this could also provide data on the specificity of their recombination, which is lacking elsewhere in the manuscript.

Comparison between astrocyte-specific cKO and global KO: Considering the abundant expression of CD38 in astrocytes compared to other cell types in the brain, I am wondering whether the comparison between the current astrocyte-specific CD38 cKO and the previous constitutive CD38 KO mice would provide a different phenotype with respect to its importance in synaptic function in neural circuits that mediate social behaviors in various brain regions. The authors note the importance of CA1, CA2, and NAC in social memory, but they only assessed synapses in mPFC. Multiple studies, including one from the authors, have reported that constitutive CD38 KO mice exhibit impaired social behaviors. Expanding beyond what is already known would require better spatial and temporal regulation of CD38 expression than presented here.

Rescue experiments: The authors claim that reduced levels of Hevin secretion are responsible for reducing intracortical synapses in mPFC and the inability of their CD38 KO ACM to stimulate synapse formation. However, Hevin has primarily been linked to the formation of VGluT2+ synapses with only a transient effect on VGluT1+ synapses. Furthermore, Hevin's synaptogenic effect in astrocyte conditioned media is masked by its homolog Sparc. To claim that Hevin is responsible for reducing VGluT1+ synapses in mPFC the authors need to do a rescue experiment by expressing hevin in CD38 KO through AAVs brains or intracortical injections of recombinant Hevin.

Other synaptogenic factors: The authors focus on Sparcl1/Hevin; however, other synaptogenic factors have been reported to affect VGluT1+ excitatory synapse formation and development directly. Notably, thrombospondins have been shown to regulate the formation of this specific synapse type through their receptor a2d1. The authors do not report any investigation into this family of factors despite their clear link to VGluT1+ synapse development.

Effect of CD38 cKO on astrocyte numbers: The authors note that CD38 cKO alters GFAP expression; however, they also report a decrease in the number of GFAP+ and S100β+ cells without a change in NDRG2+ cells. The authors should address this discrepancy in astrocyte numbers with additional known markers such as Sox9.

MBP quantification: The authors previously reported changes in MBP expression and oligodendrocyte maturation in the global CD38 KO animals. However, there is no quantification of the MBP staining in the cKO in supplementary figure 1. It would be important to verify that white matter structures developed properly in their cKO model, especially in mPFC.

Minor Concerns:

1. SPARCL1 annotation should be Sparcl1.
2. Avoid repetition of the same sentences in multiple places. E.g., The sentence- "Social behavior is essential for the health, survival, and reproduction of animals" is repeated both in the abstract and introduction.
3. The introduction needs to be thoroughly revised. In the first paragraph, a description of various studies(Fmr/Mecp2) which indicated the importance of synaptic function in neural circuits that mediate social behaviors in various brain regions could be presented later part of the introduction in a very concise manner since the article doesn't cover anything related to these genes. This part can be presented along with the narration of CD38, where authors described its importance in social behavior. Introduce the importance of social behavior and their behavioral paradigm, especially what social memory is and what brain regions are important for it.
4. Introduction feels too short and abrupt.
5. In Figures 2 and 3, Are the spine numbers/density/synapses affected in the p42 ctrl/CD38 AS-cKO group compared to the p10 ctrl/CD38 AS-cKO group?
6. In Figure 2; The authors should compare both the behavioral phenotype seen in two different tamoxifen injection/time points with the respective constitutive CD38 KO mice data.
7. In Figures 3 and 4, the authors should analyze the spine numbers/density both in WT or CD38 KO ACM treated experiments and Sparcl1 KD/Sparcl1 treated rescue experiments?
8. The discussion section needs to be revised to reflect better the conclusions drawn from the data without overstatement.

2. Significance:

Significance (Required)

Understanding the mechanisms underlying control of behaviors is important and linking non-neuronal cell types to behavioral processes is novel and timely. However, the study at its current state lacks important controls, and interpretations are overstated and often too targetted to a favorite mechanism. These concerns limit the impact of the study and reduces its significance.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

More than 6 months

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary

In their manuscript, Hattori et al., put forward evidence that the knock-out of CD38 expression in astrocytes at approximately post-natal day 10 (referred to as CD38 AS-cKO P10) leads to a specific deficit in social memory in adult mice, while other types of memory remain unaltered. Using immunohistochemistry (IHC), the authors found a reduced number of excitatory synapses in the medial prefrontal cortex (mPFC) of CD38 AS-cKO P10 mice. Switching to in vitro primary cell culture models, the authors identify the astrocyte secreted protein SPARCL1 as a relevant synaptogenic factor. Using pharmacological dissection of relevant signaling pathways, Hattori et al., propose that cADPR formation and calcium released from intracellular stores, is essential for SPARCL1 secretion from astrocytes. Finally, the authors analyzed the transcriptome of primary CD38 KO astrocytes using bulk mRNA sequencing, and found that genes related to calcium signaling were downregulated in these cells.

Major commments:

- Are the key conclusions convincing?
1. From a global perspective, the multiple lines of evidence provided by the authors strongly suggest that expression of CD38 in astrocytes is important for synaptogenesis in the mPFC of P10 mice, with ablation of CD38 and reduced synapse formation leading to social memory deficits at P70. However, the data concerning the role of astrocyte-secreted SPARCL1 is not particularly strong: further experiments are needed to support this claim (see below).
- Are the claims preliminary or speculative?
1. As it stands, there is no proof that the claimed astrocyte-specific deletion of CD38 is actually astrocyte specific. This evidence is crucial: without it the reported effects could be due to non-specific CD38 knock-out in other CNS cells. In this respect, the Western Blot in Supplementary Figure 1A does not provide information on astrocyte-specific deletion, merely that CD38 was globally reduced in the mPFC. Interestingly, the authors have previously published data (Hattori et al., 2017, 10.1002/glia.23139) showing that CD38 expression is mostly astrocyte-specific, peaking at p14, which coincides with the peak period of synaptogenesis. The degree of CD38 heterogeneity is also an issue that I think the authors need to consider. Do they information on this? Is CD38 expressed in every astrocyte of the CNS, or are there some astrocytes that are CD38 negative at P14? Is the mPFC a region specifically enriched in CD38 positive astrocytes and does this explain the observed behavioral deficit? I think if this is known, the authors should mention it in the "Introduction" or "Discussion". If this is not known, maybe the authors could provide data addressing the issue.
2. I think the authors should take more caution in claiming that SPARCL1 is the main factor secreted through the CD38 signaling pathway and responsible for increased synaptogenesis. This is for several reasons, all centered on data displayed in Figure 4 and Supplementary Figure 6:
- a) Western Blot (WB) data: The "Materials and Methods" section for WB does not indicate how protein loading and transfer efficiency were controlled for. Normalizing to β-Actin levels is an acceptable way to control for loading and transfer efficiency when using cell lysates. However, in the absence of such an abundant structural protein in conditioned media it is unclear how loading and transfer was controlled for under these conditions. Do the authors normalized the CD 38 KO AS ACM data by expressing protein levels relative to those from WT AS ACM? Is BDNF being used as a control, based on proteomics data? If so, why is proteomics data not given in the manuscript and why is this control not shown for all ACM blots? I realize that (quantitative) blotting using ACM is difficult, but I am also not convinced that the methodology used is sufficiently rigorous. Simple steps to give confidence would be Coomassie staining of gels both before and after membrane transfer, to show that i) the total protein amount loaded was the same in each lane of the gel and ii) the transfer to the nitrocellulose membrane was complete. In addition, Ponceau S staining

of the nitrocellulose membrane should also have been performed and displayed, to show (roughly) equal amounts of protein were transferred for each lane. In summary, the WB data quantification needs to be better controlled.
The values of the Y axis in these graphs (and throughout the manuscript) are simply too small to be read properly. Finally, I want to highlight the general lack of precision regarding the nature of the replication unit (the "n"). For example, the legend of Figure4C-D states "n = 6", but we have no idea if these are 6 independent primary cultures originating from 6 mice, 6 independent cultures from the same mouse, 6 repeats of the Western Blot using the same sample etc. This issue is valid for the whole manuscript: in my opinion, the authors should be more much careful when it comes to these crucial elements of scientific reporting.
- b) While the data hint at an important role of SPARCL1 in synapse formation, when the authors tested if ACM from CD38 KO astrocytes supplemented with exogenous SPARCL1 could rescue synapse formation, the effect was incomplete, with only a trend to an increase in synapse number (Figure 4J-K). Perhaps the authors simply forgot to indicate the statistical significance of differences between the experimental groups (Figure 4K)? However, if there really were no statistically significant differences observed, the authors should reduce the strength of their conclusions regarding SPARCL1. This protein may well be pro-synaptogenic but, as it stands, other factors could well be in play. Perhaps the authors should have tried higher concentrations of SPARCL1 to further boost synaptogenesis? In this respect, the SPARCL1 knockdown (KD) experiment in Supplementary Figure 6B-D is an important addition, but should be supplemented by rescue with an siRNA-resistant recombinant SPARCL1? If SPARCL1 is a major player in synaptogenesis, the prediction is that synapse numbers would be close to wild type levels with this approach.
- c) In my opinion, there are also issues with the data displayed in Figure 4H-I. The authors want to convince the reader that SPARCL1 is mostly an astrocytic protein using immunohistochemistry on mouse mPFC sections, co-labelled with antibodies against neuronal and astrocytic markers. In these panels, we are presented with images showing a few cells, in which it seems SPARCL1 is absent from NeuN positive cells, present in WT astrocytes and reduced in CD38 AS-cKO P10 astrocytes. However, the numbers of cell counted and lack of quantification severely impact on the strength of this conclusion. In my opinion, the authors should have quantified their IHC data by counting cells and establishing the ratios of SPARCL1 positive over NeuN or S100β positive cells, in both control and CD38 AS-cKO P10 animals. This experiment would provide critical information that the conditional gene targeting strategy is robust. The authors should also consider quantifying the intensity of the SPARCL1 signal in astrocytes. This is recommended as the image displayed in Figure 4I for the CD38 AS-cKO is problematic: are the authors really claiming that the reduction in SPARCL1 expression following cKO of CD38 in astrocytes is at best only partial? Is

11 days between the first tamoxifen injection and tissue fixation actually sufficient to allow for CD38 turnover? With low levels of protein turnover, the possibility exists that residual levels of CD38 are still sufficient to impact SPARCL1 levels. What would happen if there is a greater interval between tamoxifen administration and tissue recovery? Would levels of synaptogenesis be further reduced? Is this an issue of production versus secretion or a combination of factors?
3. The heatmap (Figure 5E-F) is simply too small to interpret. The color choice is also not accessible for colorblind readers. The authors might consider displaying this heatmap in a separate figure. The authors should also provide a supplementary table where all the genes detected are listed along with their respective counts. Furthermore, it is surprising that the authors only found genes being downregulated in CD 38 KO astrocytes. Were they really no genes up-regulated? The authors might also want to indicate the genes belong to each of the ontological categories listed in Figure 5F. On p. 11, Figure 5E: The authors should indicate in the main text they performed bulk RNA-sequencing and not another type of RNA sequencing (like single cell RNA sequencing for instance). The authors indicate n = 2 but we have no indications of the nature of the replicate (also see earlier comments). Please amend.
- Are additional experiments necessary?
I think supplementary experiments are essential to support the claims of the paper. Most are described in the section above, but to summarize:
1. Show data to prove that the CD38 AS-cKOP10 model is astrocyte-specific and leads to a total loss of CD38 in these cells.
2. WB data: The issue of protein loading and transfer efficiency should be dealt with. Quantifications should be revisited.
3. The authors should quantitatively analyze the different IHC performed in Figure 4H-I.
4. The authors should provide more information on their RNA sequencing data: list of genes detected with their FPKM values etc. The authors should display the RNA sequencing data in a separate figure, allowing the heatmap to be enlarged.
5. LC-MS/MS data: the authors should provide the list of all the proteins they identified in their LC-MS/MS experiment. As a supplementary table for instance?
The majority of these experiments should be able to be performed with pre-existing samples/tissue slices. If not, the experimental pipeline necessary exits and these supporting experiments should not be too burdensome.
- Data and methods presentation
Methods:
The authors need to work on this aspect of the manuscript. Most of the important details are already described, but some crucial ones are missing, while the phrasing used to describe methods is sometimes misleading. I will give some examples here, but this is not an exhaustive list. The fact that the manuscript is riddled with small mistakes, inconsistencies and/or oversights makes it difficult to read and creates a

negative impression. The whole manuscript would benefit from a thorough proof-reading, preferably by a native speaker.
1. in the "Immunohistochemistry and Synaptic Puncta Analysis" section on p. 21-22, we have no indication of which antibodies against "GFAP, NDRG2, VGlut1, PSD95, S100β, NenN(?) and SPARCL1" were used. It is standard practice to indicate the company, product number and lot number. The authors must also indicate the dilution at which they use these antibodies. On p.22, the authors write the cells were incubated with "Alexa- or Cy3-conjugated secondary antibodies". The excitation wavelengths of the Alexa dyes used need to be given.
2. The authors need to provide more details on the microscope they used. Merely writing "using a 63× lens on a fluorescence microscope" (p.23) is insufficient.
3. In the "LC-MS/MS" method the authors wrote: "Briefly, these proteins were reduced, alkylated, and digested by trypsin". I think that in the reduction and alkylation steps, chemicals other than trypsin were actually used. This sentence should be modified to reflect this.
4. p.19: "uM" is written when the authors very likely mean "μM". Please check the whole manuscript for repeat examples. I know this is often lab "short-hand", but it should be avoided in scientific publications.
5. The authors should be careful when describing their data to always indicate whether they referring to experiments performed using cultured astrocytes or not. As it stands, the text is confusing: for instance, when describing RNA-sequencing data in Figure 5, the main text appears to indicate that these astrocytes were acutely isolated from adult mice, when in fact they were obtained from primary cultures. Given concerns in the literature about potential differences between acutely isolated and cultured astrocytes (Foo et al., Neuron, 2011), this is essential.
Data presentation:
The figures appear to have been produced in a rush - and almost have a "screenshot" feel to them. This is not a scientific issue per se, but does impact on the overall impression given by the manuscript. The following is a non-exhaustive list of issues with the figures. I list the major ones that the authors should correct.
1. Almost all Y axis labels are too small. The authors should comply to the basic journal requirements in terms of font sizes. Some axes do not end on a tick (e.g. Figure 3R). This is not dramatic, but should be corrected. Globally, the authors need to display bigger bar plots - most of them are extremely hard to read. Labeling should also be checked: Figure 4K, the Y axis label indicates values displayed are in %, when I think the axis graduation displays ratio values. Some of the IHC pictures are also too small to be easily interpreted.
2. The heatmap in Figure 5E is impossible to read and, as such, has little or no value for the manuscript.
3. Scale bars: where is the scale bar in Figure 2A? Figure 3A-H: Is the scale bar really representing 10 millimeters? Supplementary Figure 3A: scale bar is missing. Please

check for similar issues throughout the manuscript.
4. Figure Legends are problematic, and often contain incorrect or incomplete information. Examples include: Supplementary Figure 1: The description of panels J, L and N appears to be missing. Please also use the Greek letter beta and not 'b' for S100β. Supplementary Figure 5: I think the term "KO" is missing after CD 38 in the legend title. Figure 3: why state that nuclei were counterstained with DAPI in Figure 3P,Q, when this precision is not given for panels Figure 3A-H? Figure 3A-H: If the authors choose to explicitly state PSD95 is a post-synaptic marker, why not indicate that VGlut1 is a pre-synaptic marker? Same issue in Supplementary Figure 4.
5. There are multiple instances of panels being wrongly referred to in the main text. On p.10, Figure 4H is referenced, when I think the authors mean Figure 4I; on p.10, Figure 4I-J are referred to when the authors clearly describe data found in Figure 4J-K. These types of mistakes are problematic and recur throughout the manuscript.
- Statistical analysis
As mentioned above, the exact nature of the replicates is often not stated, when the "n" number is indicated. The authors must correct this issue and give the information either at the appropriate point in the main text or in the figure legend.

The authors should also be more consistent in the way they indicate which statistical tests were performed. This should also be indicated either at the appropriate point in the main text or in the figure legend. Furthermore, care should be taken to ensure statistics are presented in an appropriate manner: at the end of legend for Figure 4, it is indicated #$p < 0.05$ vs. CD38 KO ACM. This hashtag symbol is completely absent from the figure. In Figure 4F-G, the lack of statistical symbols seems to indicate no statistical tests were performed on these data, when the legend covering these panels states "*$p < 0.05$ versus P70", indicating some tests were done. We cannot interpret this panel without knowing which comparisons were done exactly and which were significant.

In the "Materials and Methods", the authors give no indication that the assumptions of the statistical test they used were met (normality of data distribution for t-tests, homogeneity of variances for ANOVA...). This needs to be checked, and if not met, appropriate non-parametric tests should be used instead.

Minor commments:

- Specific experimental issues that are easily addressable.
Most of the experimental issues that need to be addressed are given in previous sections and should be easily addressable.
- Citation of previous studies?
Adequate

- Clarity and accuracy of text and figures
There are issues with the clarity and accuracy of text and figures - which are described above. The text is also often problematic in its phrasing and other, more fundamental aspects. For instance, the authors spent a considerable amount of time speaking about the role of oxytocin, when they only performed one measurement of oxytocin levels in mice.
- Suggestions to improve the presentation of data and conclusions?
All my suggestions to improve the presentation of data can found in previous sections. As for improving the authors presentation of their conclusions, the authors should make a considerable re-drafting effort, particularly for the "Discussion", which lacks clarity in how supporting arguments are built and presented. For example, on p.13, I am confused with the argument made by the authors. Their data are focused on synapses onto pyramidal neurons of the mPFC, but here the discussion states that the behavioral phenotype they observed in CD38 AS-cKOP10 might be explained by a lack of mPFC neurons synapsing onto neurons in the Nucleus Accumbens (assuming that "NAc" really refers to this brain region, as the definition is missing from the text). I think the authors should make it clear if this is their interpretation of their own result, which essentially renders their focus on mPFC pointless, or a speculation on possible other mechanisms that could also explain their behavioral results. Personally, given the data shown, I believe the authors should focus on explaining how their data in mPFC might explain the behavioral output observed. The authors could also provide perspectives on how the hypothesis laid down in this paragraph would be tested. When the authors write on p.14 "We identified SPARCL1 as a potential molecule for synapse formation in cortical neurons" why use the word "potential"? Does this mean the authors consider their data on SPARCL1 (one of the key messages of the paper) invalid? If the authors themselves think the role of SPARCKL1 is ambiguous based on their own data, they should perform further experiments. P. 13, the authors write: "Moreover, many studies have shown that astrocyte-specific molecules, including extracellular molecules such as IL-6, are involved in memory function"; Interleukin 6 (Il-6, abbreviation not defined in the manuscript) is definitely not an astrocyte-specific molecule (see, for example, Erta et al., 2021 10.7150/ijbs.4679).

2. Significance:

Significance (Required)

NATURE AND SIGNIFICANCE OF THE ADVANCE:
I think that despite the issues described above, this manuscript, once revised, could have a strong impact in the field. It would fuel the current paradigm shift which puts astrocytes at the forefront of neuronal circuit wiring during development with links to

adult behavior. By identifying clear molecular targets involved in astrocyte-driven synaptogenesis, this article could help the clinical field to find new druggable targets, which may help reverse aging-related cognitive decline.

COMPARISON TO EXISTING PUBLISHED KNOWLEGDE:
This work adds new data in the specific and growing line of research that study how astrocytes control synaptogenesis. Recent reviews have summarized advances in this field (Shan et al., 2021, 10.3389/fcell.2021.680301; Baldwin et al., 2021, 10.1016/j.conb.2017.05.006).

AUDIENCE:
Neuroscientists in general, clinicians interested in cellular and molecular causes of neurodevelopmental disorders leading to social dysfunctions.

REVIEWER EXPERTISE:
Astrocyte biology; Astrocyte-neuron interactions and synapse assembly; Neuronal circuit formation and plasticity

Referees cross-commenting

After careful reading of the other comments, I feel that there is considerable agreement/overlap between the reviewers on the main issues with this manuscript. Perhaps the major difference relates to the amount of further work necessary for the manuscript to be publication ready.

As Reviewer 3 rightly points out, this is always a moot point: how much is it reasonable for reviewers to ask authors to do? While I agree with all of Reviewer 1's comments regarding the rigour of the mass-spec/western blot analysis, it seems to me that from a molecular/cell biological point of view, the key issue is whether Sparcl1 is a synaptogenic factor released from astrocytes following CD38/cADPR/calcium signaling (irrespective of whether other factors may be in play); and whether raising Sparcl1 levels is sufficient to recover spine morphology and synapse numbers. Of course, if these experiments were performed in vivo using AAV-mediated overexpression of Sparcl1, it is also reasonable to think that the deficit in social memory may be reversed on testing.

The issues of whether there is a difference in observable behavioral phenotypes between the astrocyte-specific and constitutive CD38 knock-outs is an interesting one, as is why there is only a deficit in social memory seen following astrocyte-specific CD38 ablation. These issues should at least be discussed.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Hattori et al. assessed the role of astrocytic CD38 by generating astrocyte-specific conditional CD38 knockout mice and discovered defects in social memory, synapse, and spine density in the mPFC. They further showed that conditioned media from CD38-deficient astrocytes are defective in promoting synapse formation. A known astrocyte-derived synapse promoting protein, Sparcl1, is reduced in the conditioned medium from CD38 KO astrocytes and pharmacological experiments suggest that CD38 and calcium signaling regulates Sparcl1 secretion by astrocytes.

The discoveries are novel and important and will be of broad interest to readers. However, the following concerns need to be addressed to improve the manuscript.

Major comments:

1. It's unclear if experiments were conducted while the experimenters are blinded to the genotype of the mice. This is essential for behavior tests.
2. Hippocampus is also important for memory formation. Do synapse and spine densities change in the hippocampus?
3. The proposed model of CD38 inducing Ryr3-mediated calcium release from internal stores is interesting. However, the Barres database showed that Ryr3 is not expressed by mouse astrocytes. Could the authors demonstrate the presence of Ryr3? That's a key link in their model that hasn't been demonstrated to operate in astrocytes.
4. The authors demonstrated reduced synapse and spine density in mPFC.

Interestingly, a battery of behavior tests showed no defect, except for the social memory test. Reducing synapses in mPFC should affect a range of behaviors. Why that is not the case here?
5. The authors only tested very short-term memory (30 minutes delay). Does CD38 regulate long-term memory? It would be important to know but I realize that a single paper cannot address all questions and therefore do not think addressing this point is a prerequisite for publication.

Minor comments:

1. Fig. 2F, multiple comparison adjustment is needed.
2. Fig. 3A, scale bar is 10 micrometers, not millimeters
3. Fig. 4C, D, it is unclear if the quantification is normalized to actin loading control. BDNF levels appear lower in KO, though not significantly different, raising the question of whether an equal amount of samples was loaded.
4. Need to validate whether CD38 levels are reduced in P42-46-injected adult knockout before concluding that CD38 is required only during development

2. Significance:

Significance (Required)

Astrocytic contribution to social memory has not been reported. This study is thus the first report on the role of astrocytes in social memory. Their discovery of CD38-regulation of Sparcl1 release is also novel and important for synapse formation, although more evidence is needed to support this point (see major comments above). This study will be of broad interest to neuroscientists. I have expertise in cellular and molecular neurobiology and can evaluate all parts of the paper.

Referees cross-commenting

I agree with the issues that the other reviewers pointed out, especially the need for improving data reporting and consistency/accuracy. Overall, I think this manuscript has potential and the issues are addressable.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

Revision Plan

Manuscript number: RC-2021-01217
Corresponding author(s): Tsuyoshi, Hattori

1. General Statements [optional]

The goal of our study is understanding the mechanism underlying control of behaviors by glial cells. By reflecting the reviewer's insightful suggestions, we would like to clarify roles and mechanisms of astroglial CD38-mediated synapse formation and social behavior.

2. Description of the planned revisions

We plan to conduct 6 following major experiments and revision of the Discussion section to address the points raised by the reviewers. Especially, the reviewers commented about contribution of SPARCL1 on synaptogenesis under CD38/cADPR signaling. By conducting plan 2), we will clarify this issue.

1) Confirmation of astrocyte-specific deletion of CD38 in CD38 AS-cKO^{P10} mice

(Reviwer#2) *"As it stands, there is no proof that the claimed astrocyte-specific deletion of CD38 is actually astrocyte specific. This evidence is crucial: without it the reported effects could be due to non-specific CD38 knock-out in other CNS cells. In this respect, the Western Blot in Supplementary Figure 1A does not provide information on astrocyte-specific deletion, merely that CD38 was globally reduced in the mPFC. Interestingly, the authors have previously published data (Hattori et al., 2017, 10.1002/glia.23139) showing that CD38 expression is mostly astrocyte-specific, peaking at p14, which coincides with the peak period of synaptogenesis. The degree of CD38 heterogeneity is also an issue that I think the authors need to consider. Do they information on this? Is CD38 expressed in every astrocyte of the CNS, or are there some astrocytes that are CD38 negative at P14? Is the mPFC a region specifically enriched in CD38 positive astrocytes and does this explain the observed behavioral deficit? I think if this is known, the authors should mention it in the "Introduction" or "Discussion". If this is not known, maybe the authors could provide data addressing the issue."*

As the reviewer suggested, astrocyte-specific deletion of CD38 in our model is a critical point of our study. We used Glast^{CreERT2} mice to generate CD38 AS-cKO^{P10} mice. The specificity of this Glast^{CreERT2} mice was already reported as 84.9% of s100b positive astrocytes, 8.1% of CC1 positive oligodendrocytes, and 1.6% of NeuN positive astrocytes, when tamoxyfen was administered at adult stage (Mori et al., 2006). To confirm astrocyte-specific deletion of CD38 in our CD38 AS-cKO^{P10} mice, we will perform in situ hybridization or immunohistochemistry of CD38 in CD38 AS-cKO^{P10} at P14 to P20. At the same time, we will examine CD38 expression

not only in the mPFC but also hippocampus, to clarify whether CD38 is specifically expressed in astrocytes of the mPFC or not.

2) Rescue experiments of synapse formation in CD38 AS-cKO^{P10} mice by injection of SPARCL1

(Reviewer#1) *"Rescue experiments: The authors claim that reduced levels of Hevin secretion are responsible for reducing intracortical synapses in mPFC and the inability of their CD38 KO ACM to stimulate synapse formation. However, Hevin has primarily been linked to the formation of VGluT2+ synapses with only a transient effect on VGluT1+ synapses. Furthermore, Hevin's synaptogenic effect in astrocyte conditioned media is masked by its homolog Sparc. To claim that Hevin is responsible for reducing VGluT1+ synapses in mPFC the authors need to do a rescue experiment by expressing hevin in CD38 KO through AAVs brains or intracortical injections of recombinant Hevin."*

To investigate whether supplementation of SPARCL1 is sufficient for recovering the reduced synapse formation in CD38 AS-cKO^{P10}, we will perform injection of recombinant SPARCL1 or infection of SPARCL1-expressing AAV to the mPFC of CD38 AS-cKO^{P10} and evaluate recovery of excitatory synapse numbers by staining of Vglut1 and PSD95. Furthermore, we found SPARCL1 expression was decreased in cultured CD38 KO astrocytes. We will examine other synaptogenesis-related factors (Thrombospondin1, TNFa, TGFb, BDNF, GPC4&6, sparc) in these cells.

3) Evaluation of synapse formation in other social memory-related regions.

(Reviewer#3) "*Hippocampus is also important for memory formation. Do synapse and spine densities change in the hippocampus?"*

As the reviewer commented, hippocampus as well as mPFC plays a critical role for social memory formation, especially ventral CA1 and dorsal CA2 (Okuyama et al., 2016, Oliva et al., 2020). To examine effect of astroglial CD38 deletion on synapse formation in these area, we plan to perform immunohistochemical analysis of synaptic proteins (Vglut1 and PSD95) in the CA1 and CA2 of adult CD38 AS-cKO^{P10} mice.

4) Control experiment of synapse analysis in vitro

(Reviewer#1) "*Synapse analysis in vitro: The authors are missing key experimental controls for their analysis of synapse induction by astrocyte conditioned media. Firstly, the authors do not include a condition of neurons cultured alone without astrocytes or astrocyte conditioned media treatments. This is critical to this experiment because, without this control, it is impossible to assess the effectiveness of the astrocyte conditioned media or any recombinant protein*

treatments on synapse formation. Secondly, the authors give very few details and no supporting data about the purity of their neuronal cultures. This is critical to this experiment because any contaminating astrocytes in their cultures could severely skew the data for any given condition. Finally, the authors do not specify how they determined the doses for astrocyte conditioned media and Hevin treatments. The researchers give no details on how the astrocyte conditioned media was collected or treated before adding onto neurons. For this experiment to be viable, the researchers must collect the conditioned media in serum-free media, determine the protein concentration of their samples, and the dose-response to the astrocyte conditioned media must be performed to determine the optimal dose for each batch. When comparing between genotypes, this type of quality control is critical to assess whether there is, in fact, a difference in their synaptogenic capacity. "

As the reviewer point out, synapse formation of neuron culture without ACM is important as negative control, to know the effect of ACM on synapse formation. We will perform WT and CD38 KO ACM experiment with this negative control. And we will examine and describe purity of neuron primary culture at 14 DIV in the Materials and Methods section. Furthermore, we will describe detailed protocol of ACM experiments.

5) Investigation of astrocyte number in CD38 cKO.

(Reviewer#1) "*Effect of CD38 cKO on astrocyte numbers: The authors note that CD38 cKO alters GFAP expression; however, they also report a decrease in the number of GFAP+ and S100β+ cells without a change in NDRG2+ cells. The authors should address this discrepancy in astrocyte numbers with additional known markers such as Sox9."*

We reported that systemic deletion of CD38 does not change he number of astrocytes but decreases GFAP, s100b and Cx43 expression (Hattori et al., 2017). In the present study, the number of NDRG2 positive astrocytes were also not changed in CD38 AS-cKO^{P10} (Supplementary Figure 1). Because NDRG2 is used as a marker of protoplasmic and fibrous astrocytes (Flugge et al., 2014), we conclude number of astrocytes is not changed in CD38 AS-cKO^{P10}. To confirm this result, we will further examine number of astrocytes in the mPFC of CD38 AS-cKO^{P10} at P20 by using sox9 antibody.

6) Investigation of ryanodine receptor 3 (RYR3) expression in astrocytes

(Reviewer#3) *"The proposed model of CD38 inducing Ryr3-mediated calcium release from internal stores is interesting. However, the Barres database showed that Ryr3 is not expressed by mouse astrocytes. Could the authors demonstrate the presence of Ryr3? That's a key link in their model that hasn't been demonstrated to operate in astrocytes."*

Revision Plan

As the reviewer suggested, we proposed that RYR3 is a candidate molecule as downstream protein of CD38/cADPR signaling pathway in astrocytes. We will provide information of RYR3 and other RYRs expression in astrocytes by qPCR and immunostaining of cultured astrocytes or developing brain or using public database of RNA-seq.

3. Description of the revisions that have already been incorporated in the transferred manuscript

Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript. If no revisions have been carried out yet, please leave this section empty.

Reviewer#1

1) *"Synapse analysis in vivo: For the analysis of VGluT1 excitatory synapses in mPFC, it is not clear how the statistical analysis was performed. From the plotted error bars, it seems that the investigators used individual z-projections as the n for a t-test. This is inappropriate for this analysis as it would overinflate the N and down the p-value. It would be more appropriate to plot and compare animal averages between conditions or use a test that can account for the fact that there are repeated measures taken from the same animal. Additionally, the authors note a decrease in VGlut1+ puncta in the global CD38 KO but no change in the protein levels in both the global and cKO."*

We apologize for incorrect description of the "N" of synapse analysis in vivo (Figure 3A-K, Supplementary Figure 4A-K). We analyzed 5 confocal z-stacks per animal and compared animal average per condition. Thus, we revised previous " n = 5 z-stacks per animal, 4 animals per genotype." to " n = 4 animals per genotype." In the Figure legends of Figure 3 and Supplementary Figure 4. We also revised and described detailed method of synaptic analysis in immunohistocheimstry and synaptic puncta analysis in the Materials and Methods.

2) *"Western blots: All western blot quantification of astrocyte conditioned media should include total protein normalization. The authors do not describe how they normalize the astrocyte conditioned media blots, but without a total protein stain to normalize, it is impossible to be sure the same amount of protein was loaded into the gel for each lane. In Figure 3L, the western blot data showing the expression of VGluT1 and PSD95 should be improved, and a better representation is recommended. It is also strange that the CD38 cKO has no expression because CD38 is also expressed in endothelial cells. Why not isolate astrocytes from CD38 KO? Also, for VGluT1 and PSD95 western blots, it would be better to test mPFC lysates rather than whole cortical lysates."*

As the reviewer commented, when we quantify and compare amount of SPARCl1 protein in astrocyte conditioned media (ACM), amount of total protein loading is a critical point. Following the reviewer's suggestion, we performed Coomassie Brilliant Blue (CBB) staining of gels loaded by equal amount of protein of WT and CD38 KO ACM (Figure 4A), control, 8Br-cADPR, 2APB and Dantrolen treated ACM (Figure 5B), or control-siRNA and SPARCL1-siRNA treated ACM (Supplementary Figure 6). Then, we measured amount of each protein from CBB staining and quantified SPARCL1 protein normalized by the protein amount. We added CBB stain images and quantified data in Figure 4A, 5B, and Supplementary Figure 6.

We replaced images of Vglut1 and PSD95 to more representative ones in Figure 3L.

In CD38 AS-cKO^{P10} mice, CD38 expression at protein and mRNA level is 70-80% lower than control mice (Supplementary Figure 1B). However, its expression in CD38 AS-cKO^{P10} is significantly higher than constitutive CD38 KO mice, suggesting remaining CD38 expression in neurons or endothelial cells.

All the brain samples collected for western blot were from the mPFC. We described this in western blot analyses in the Materials and Methods.

3) *"Mass spectrometry: There is no information about how many samples were used for mass spectrometry. This reviewer is concerned that this experiment may be underpowered given that other published datasets have identified significantly more proteins in wild-type ACM (about double than what was identified here). There needs to be a quality assessment of the ACM to help ensure the production protocol can capture the full extent of proteins secreted by cultured astrocytes."*

We apologize for lack of information of N in mass spectrometry. We added "n = 2 independent cultures from 2 animals per genotype." in the Legend of Table 1. We performed mass spectrometry after measuring protein concentration of each samples from WT and CD38 KO ACM for adjusting total amount of protein. Furthermore, we also used samples from neurobasal medium as negative control to confirm the identified proteins were derived from astrocyte. Thus, we think our proteomics rightly identified difference of protein level between WT and CD38 KO ACM. We revised Comparative Shotgun Proteomics using nano-liquid chromatography mass spectrometry in the Materials and Methods and the Legend of Table 1.

4) *"Other synaptogenic factors: The authors focus on Sparcl1/Hevin; however, other synaptogenic factors have been reported to affect VGluT1+ excitatory synapse formation and development directly. Notably, thrombospondins have been shown to regulate the formation of this specific synapse type through their receptor a2d1. The authors do not report any investigation into this family of factors despite their clear link to VGluT1+ synapse development.."*

5) *"MBP quantification: The authors previously reported changes in MBP expression and oligodendrocyte maturation in the global CD38 KO animals. However, there is no quantification of the MBP staining in the cKO in supplementary figure 1. It would be important to verify that white matter structures developed properly in their cKO model, especially in mPFC. "*

Revision Plan

Following the reviewer's suggestion, we measured intensity of MBP in control and CD38 AS-cKO^{P10} mice. Data was added to Supplementary Figure 1P.

Reviewer#2

1) *"WB data: The issue of protein loading and transfer efficiency should be dealt with. Quantifications should be revisited."*

As we respond to the Reviewer #1 comment 2), following the reviewer's suggestion, we performed Coomassie Brilliant Blue (CBB) staining of gels loaded by equal amount of protein of WT and CD38 KO ACM (Figure 4A), control, 8Br-cADPR, 2APB and Dantrolen treated ACM (Figure 5B), or control-siRNA and SPARCL1-siRNA treated ACM (Supplementary Figure 6). Then, we measured amount of each protein from CBB staining and quantified SPARCL1 protein normalized by the protein amount. We added CBB stain images and re-quantified data in Figure 4A, 5B, and Supplementary Figure 6).

2) *"The authors should quantitatively analyze the different IHC performed in Figure 4H-I."*

We quantified the number of SPARCL1/S100b or SPRCL1/NeuN double positive cells in the mPFC of ctrl^{P10} and CD38 AS-cKO^{P10} mice. In revised Figure 4G and H, it is shown that SPARCL1 is predominantly expressed in s100b positive astrocytes and SPARCL1/s100b double positive cells were significantly decreased in CD38 AS-cKO^{P10} mice.

3) *"The authors should provide more information on their RNA sequencing data: list of genes detected with their FPKM values etc. The authors should display the RNA sequencing data in a separate figure, allowing the heatmap to be enlarged."*

We plan to submit our RNA-seq data of CD38 KO astrocytes public database (GEO). To clearly visualize number of up and down regulated genes and pathways, we made new separated figure 6 for RNA-seq, which includes heatmap (Figure 6A), scatter pot (Figure 6B), and pathway analysis of up-regulated and down-regulated genes in CD38 KO astrocytes (Figure 6C and D).

4) *"LC-MS/MS data: the authors should provide the list of all the proteins they identified in their LC-MS/MS experiment. As a supplementary table for instance?"*

As the reviewer suggested, we added proteomics data of WT and CD38 KO astrocyte conditioned medium, which include accession number, protein name, Abundance Ratio (CD38 KO ACM/WT ACM), and Abundance Ratio variety in Supplementary table 1.

5) The reviewer pointed out extensively concerning the Data and Methods presentation and the Statistical analysis. We revised all the points in the Materials and Methods section, Figures, and Figure Legends.

Revision Plan

Reviewer#3

1) “It's unclear if experiments were conducted while the experimenters are blinded to the genotype of the mice. This is essential for behavior tests.”

We apologize for lack of the experimental protocol. We added “Researchers were blind to genotypes during experiments.” in Three chamber test in the Materials and Methods.

4. Description of analyses that authors prefer not to carry out

Reviewer#1

1) *“RNA sequencing: RNA sequencing results seem underpowered, and an accurate description of their collection methods is missing. It also seems to this reviewer that any prolonged culturing of the astrocytes would lead to additional transcriptional changes independent of their genetic manipulation. To avoid confounds due to culture artifacts, it might be cleaner to FACS sort astrocytes using a fluorescent reporter such as the Aldh1l1-eGFP line or RTM in their GLAST-creERT2 model. In the latter case, this could also provide data on the specificity of their recombination, which is lacking elsewhere in the manuscript.”*

As the reviewer suggested, these methods using Aldh1l1-eGFP or RTM of Glast^{CreERT2} are useful tools to isolate astrocytes directly from the developing mPFC. We isolated mRNA for RNA-seq from the cells at 5DIV before cells become confluent, and on this condition cell morphology and cell concentration of WT and CD38 KO astrocytes were not different. Thus, we think that few possibility of prolonged culturing independent of genotype affected results of our RNA-seq analyses. However, we are interested in examining effect of glial CD38/cADPR signaling on gene expression at each stage of postnatal brain development. We would like to perform these in vivo RNA-seq analysis in future study.

1st Editorial Decision **29th Mar 2022**

Dear Dr. Hattori,

Thank you for submitting your manuscript to The EMBO Journal. This submission was reviewed at Review Commons and submitted to The EMBO Journal.

I have now had a chance to take a look at it, the referee comments and your point-by-point response. I appreciate the reported findings as well as your response. I would therefore like to invite a revision addressing the concerns raised as outlined in your response.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

I thank you for the opportunity to consider your work for publication. Let me know if we need to discuss anything further

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

I have attached a PDF with helpful tips on how to prepare the revised version

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (27th Jun 2022). However, I can easily extend the revision time. Please discuss the revision progress with me around the 3 months timeframe.

We are very grateful to all the reviewers for their critical comments and useful suggestions that have helped us improve our manuscript. In response to the comments by the reviewers, we made the following major revisions:

1) We confirmed astrocyte-specific deletion of CD38 in CD38 AS-cKO^{P10} mice by MACS-based astrocyte isolation (Fig EV1):

2) We confirmed a role of SPARCL1 in astroglial CD38-mediated synaptogenesis by rescue experiments in vitro and in vivo using SPARCL1 recombinant protein (Fig 5A-D):

3) We evaluated synapse formation in other social memory-related regions such as the CA1 and CA2 of the hippocampus of CD38 AS-cKO^{P10} mice (Fig EV5):

4) We re-evaluated SPARCL1 level in astrocyte conditioned medium by CBB staining-based normalization (Fig 4A-B and Fig 6B-C).

5) We analyzed again synapse formation of cultured neurons in WT or CD38 KO ACM with a negative control (Fig 3I and J).

6) We quantified the number of SPSRCL1-positive astrocytes in CD38 AS-cKO^{P10} mice (Fig 4G and H).

In our point by point reply to the comments of the reviewers below we described other experiments and analyses we have done. We hope our revisions and responses address all concerns raised.

Reviewer #1

In their work submitted for review, Hattori et al. identify an astrocyte enriched protein (CD38) as important for social memory tasks in mice. The authors developed a conditional KO model to remove CD38 specifically in astrocytes using the GLAST-CreERT2 line crossed to a CD38 floxed line. The investigators use a three-chamber social approach test to show that loss of CD38 leads to reduced interaction time with a novel social stimulus only when the animal is given a break between test periods. The authors test whether changes in neuronal morphology or

synapses in the medial prefrontal cortex (mPFC), a region important for social memory, can account for their behavioral phenotype. The researchers found that mPFC neurons in their conditional CD38 KO (cKO) animals have significantly less mature spines than wild-type (WT) controls. The authors then claim that this reduction in mature spines correlates with a reduction in VGluT1 positive excitatory synapse density in mPFC of cKO vs. WT. Next, the investigators use mass spectrometry of astrocyte conditioned media and neuronal cultures treated with astrocyte conditioned media to test whether a known astrocyte secreted synaptogenic factor, Sparcl1/Hevin, could underlie their reported changes in synapse density in their cKO animals. Finally, the authors use pharmacological inhibitors against different components of the CD38 signaling pathway to test whether CD38 regulates Hevin secretion by astrocytes. While the reported behavioral phenotype is interesting, this reviewer has several major concerns with the data claiming that reduction in Sparcl1/Hevin is underlying synaptic phenotypes in the CD38 cKO. Therefore, the paper is not suitable for publication without addressing the concerns listed below.
Major Concerns:

Comment#1
Synapse analysis in vivo: For the analysis of VGluT1 excitatory synapses in mPFC, it is not clear how the statistical analysis was performed. From the plotted error bars, it seems that the investigators used individual z-projections as the n for a t-test. This is inappropriate for this analysis as it would overinflate the N and down the p-value. It would be more appropriate to plot and compare animal averages between conditions or use a test that can account for the fact that there are repeated measures taken from the same animal. Additionally, the authors note a decrease in VGlut1+ puncta in the global CD38 KO but no change in the protein levels in both the global and cKO.

We apologize for incorrect description of "*n*" of *in vivo* synapse analysis. According to the Reviewer's comment, we re-analyzed 5 confocal z-stacks per animal, compared average of animals per condition and increased the number of mice for the synapse analysis. We confirmed significant decrease of synapse number in CD38 AS-cKO^{P10} mice (Fig 3D). We revised previous description such as "n = 5 z-stacks per animal, 4 animals per genotype." to "n = 5 animals per genotype.", in the Figure legend of Figure 3B-D. We also analyzed synapse number of Fig EV5, Appendix FigS2 and Fig S3 in the same manner. We described detailed method of synaptic analysis in "Immunohistocheimstry and synaptic puncta analysis" in the Materials and Methods

(line 629-648).

As the reviewer commented, although in CD38 AS-cKO^{P10} mice the number of VGlut1 puncta was not changed (Fig 3B), constitutive CD38 KO mice showed decreased number of VGlut1 puncta in the mPFC (Appendix Fig S3B). Furthermore, the number of primary dendrite of the pyramidal neuron (Appendix Fig S1B) in the mPFC of constitutive CD38 KO mice were also slightly lower than those of CD38 AS-cKO^{P10} mice (Fig 2B). We think that these slight differences may be due to neuronal CD38 or astrocytic CD38 expressed earlier than P10. Because CD38 is expressed in the cortical neurons and constitutive CD38 KO mice exhibit abnormal development of astrocytes at P1 (Hattori et al., 2017, GLIA). The comparison between constitutive CD38 KO and CD38 AS-cKO^{P10} mice was described in the Discussion section (line 397-411).

Comment#2

Synapse analysis in vitro*: The authors are missing key experimental controls for their analysis of synapse induction by astrocyte conditioned media. Firstly, the authors do not include a condition of neurons cultured alone without astrocytes or astrocyte conditioned media treatments. This is critical to this experiment because, without this control, it is impossible to assess the effectiveness of the astrocyte conditioned media or any recombinant protein treatments on synapse formation. Secondly, the authors give very few details and no supporting data about the purity of their neuronal cultures. This is critical to this experiment because any contaminating astrocytes in their cultures could severely skew the data for any given condition. Finally, the authors do not specify how they determined the doses for astrocyte conditioned media and Hevin treatments. The researchers give no details on how the astrocyte conditioned media was collected or treated before adding onto neurons. For this experiment to be viable, the researchers must collect the conditioned media in serum-free media, determine the protein concentration of their samples, and the dose-response to the astrocyte conditioned media must be performed to determine the optimal dose for each batch. When comparing between genotypes, this type of quality control is critical to assess whether there is, in fact, a difference in their synaptogenic capacity.

1) To confirm the effect of ACM on synaptogenesis, we performed again in vitro synaptogenesis assay with neurons cultured without ACM. We cultured primary neurons in non-conditioned culture medium, WT- or CD38 KO-ACM and quantified synapse number by immunocytochemistry of VGlut1 and PSD95. We found significantly higher

synapse number in neurons in WT ACM than those of neurons without ACM. This result is shown in Figure 3I and J and described in the Results section (line 235-242).
2) We examined purity of neurons by immunocytochemistry using NeuN antibody. The purity of neurons in WT and CD38 KO ACM was 98.2 ± 0.9 % and 98.5 ± 0.2 %, respectively. We mentioned this in "Primary neuron culture with conditioned medium from astrocyte cultures" of the Material and Methods section (line 551-553).
3) We apologize for insufficient description regarding collection method of ACM. We described as follows

"Astrocytes were replated in 10 cm diameter dishes at the density of 5×10^5 cells per dish after 9 days of cultivation and then cultured these cells for 48 h. Then all of the media was replaced with Neurobasal medium supplemented with 2% B27 supplement and 0.5mM L-glutamine after washing with Phosphate-buffer saline (PBS) 3 times. The media were harvested 24 h after replacement, and filtrated by Millex-GP filter (SLGPR33RS, Merck Millipore) and centrifuged at 3000 g for 3 min to remove cellular debris. 500 μl of the collected medium was concentrated with Amicon Ultra 10kDa (UFC501008). Total protein concentration was determined by Bradford assay and protein concentration of each ACM sample was adjusted by adding Neurobasal medium supplemented with 2% B27 supplement and 0.5mM L-glutamine."

We added the detailed collecting methods in "Primary neuron culture with conditioned medium from astrocyte cultures" (line 534-544) and "Western blot analyses" (line 586-589) in the Materials and Methods section.

Comment#3

Western blots: All western blot quantification of astrocyte conditioned media should include total protein normalization. The authors do not describe how they normalize the astrocyte conditioned media blots, but without a total protein stain to normalize, it is impossible to be sure the same amount of protein was loaded into the gel for each lane. In Figure 3L, the western blot data showing the expression of VGluT1 and PSD95 should be improved, and a better representation is recommended. It is also strange that the CD38 cKO has no expression because CD38 is also expressed in endothelial cells. Why not isolate astrocytes from CD38 KO? Also, for VGluT1 and PSD95 western blots, it would be better to test mPFC lysates rather than whole cortical lysates.

We thank the reviewer for valuable suggestion. As the reviewer commented, total protein loading of astrocyte conditioned media (ACM) is critical for comparison of

amount of specific proteins between different samples. We performed Coomassie Brilliant Blue (CBB) staining of gels loaded with equal amount of protein of WT and CD38 KO ACM (Figure 4A), control, 8Br-cADPR, 2APB and Dantrolen treated ACM (Figure 6B), or control- and SPARCL1-siRNA treated ACM (Appendix Figure S5A). Then, we normalized immunoblotting of each protein by the quantified CBB staining. By this normalization, we confirmed the decreased SPARCL1 in ACM by deletion of astroglial CD38 (Fig 4A and B), blocking of cADPR/calcium signaling (Fig 6B and C) and knockdown of astroglial SPARCL1 (Appendix Figure S5A). We described these procedure in "Western blot analyses" of the Materials and Methods section (line 606-610), and added images of CBB staining to show total amount of protein as described above.

We replaced images of Vglut1 and PSD95 to more representative images in Fig 3E.

To confirm astrocyte-specific deletion of CD38 in CD38 AS-cKO^{P10}, we isolated astrocyte from the PFC and hippocampus of CD38 AS-cKO^{P10} mice at P20 by magnetic-activated cell sorting (MACS) with the astrocyte surface antigen ACSA2 as previously performed (Holt *et al*, 2019; Kantzer *et al*, 2017; Ohlig *et al*, 2021). We confirmed successful isolation of astrocytes and found 86.5% (PFC) and 88.2% (Hippocampus) of reduction of *Cd38* mRNA expression in astrocyte fraction of CD38 AS-cKO^{P10} mice. In contrast, *Cd38* mRNA expression was not changed in ACSA2 negative fraction of the cKO mice (Fig EV1). These results indicate astrocyte-specific deletion of CD38 in the cKO mice and are described in the Results section (line 122-132).

We apologize for lack of information of sample collection. All the brain samples collected for western blot analysis were from the mPFC. We added this information in "western blot analyses" of the Materials and Methods section (line 589).

Comment#4

Astrocyte morphogenesis: Since the astrocyte-specific deletion of CD38 from P10 impairs postnatal development of astrocytes, the authors should investigate if the impaired synaptogenesis seen in later stages is due to impaired astrocyte morphogenesis or the defect in the secretion of synaptogenic proteins like Sparcl1/Hevin or thrombospondins.

To investigate impairment of astroglial development in CD38 AS-cKO^{P10} mice, we

examined expression of astrocyte-specific proteins in the developing mPFC at P20 by western blotting and immunohistochemistry analyses. We found that some of the specific proteins such as GFAP, s100β, and Cx43 were significantly decreased in CD38 AS-cKO^{P10} mice (Fig EV2). Furthermore, we found that the number of SPARCL1 positive astrocytes was significantly decreased in CD38 AS-cKO^{P10} mice at P20 (Fig 4G and H). These results indicate that deletion of astroglial CD38 impairs development of astrocytes in the postnatal brain at P20 and are described in the Results section (line 136-150 and 268-273).

Comment#5

***Mass spectrometry*: There is no information about how many samples were used for mass spectrometry. This reviewer is concerned that this experiment may be underpowered given that other published datasets have identified significantly more proteins in wild-type ACM (about double than what was identified here). There needs to be a quality assessment of the ACM to help ensure the production protocol can capture the full extent of proteins secreted by cultured astrocytes.**

We apologize for insufficient description regarding the procedure of proteomics analysis. We described the number of this experiment as "n = 2 independent cultures from 2 animals per genotype" in the Legend of Table 1. We also revised all the description of number of experiments in other experiments.

As the reviewer commented, the number of identified proteins in this study by proteomics was fewer than those in other studies (Dowell et al., 2009, J Proteome Res.; Greco et al., 2010, J Proteome Res.). Although our method of cell culture and collecting ACM were same as those of other studies, concentration method of ACM (Trichloroacetic acid-sodium lauroyl sarcosinate (TCA-NLS)) or type of capillary column used for LC-MS/MS may be the reason for this difference. Thus, there is a possibility that we did not detect all the proteins secreted from WT and CD38 KO astrocytes. However, we found that SPARCL1 as a decreased protein in CD38 KO ACM by proteomics analysis. We confirmed significant decreased of SPARCL1 and unchanged thrombospondin1 and BDNF protein in CD38 KO ACM (Fig 4A and B). Furthermore, treatment of SPARCL1 in vitro (Fig 5A and B) and in vivo (Fig 5C and D) significantly recovered the impaired synaptogenesis by deletion of astroglial CD38. Thus, we concluded that astroglial CD38 promotes synapse formation mainly through SPARCL1. However, these recovery rate were 87.9% ± 4.6 % (in vitro) and 87.1% ± 0.09 (in vivo) of controls. It is still possible that other known or unknown proteins are

involved in CD38-mediated synaptogenesis. We described the detailed methods in "Comparative Shotgun Proteomics using nano-liquid chromatography mass spectrometry" in the Material and Methods section (line 665-689) and the possibility of the involvement of other synaptogenic proteins in the Discussion section (line 426-439). Furthermore, we added the full list of identified proteins by the proteomics in Table EV1.

Comment#6

***RNA sequencing*: RNA sequencing results seem underpowered, and an accurate description of their collection methods is missing. It also seems to this reviewer that any prolonged culturing of the astrocytes would lead to additional transcriptional changes independent of their genetic manipulation. To avoid confounds due to culture artifacts, it might be cleaner to FACS sort astrocytes using a fluorescent reporter such as the Aldh1l1-eGFP line or RTM in their GLAST-creERT2 model. In the latter case, this could also provide data on the specificity of their recombination, which is lacking elsewhere in the manuscript.**

We apologize for insufficient description regarding the collecting method of RNA-seq samples. We described this procedure as follows "WT and CD38 KO astrocytes were cultured in the culture media containing 10% fetal bovine serum for 5 days. The cells were washed with PBS three times and then collected. Total RNAs were extracted from the cells using the FASTGene^{TM} RNA Basic Kit. (FG-80250, Nippon Genetics Co., Ltd)." in the Materials and Methods section (line 707-710).

As the reviewer commented, to obtain astrocytic gene profile which is similar with those in the brain, FACS-based cell isolation from CD38 AS-cKO^{P10} mice with astrocytic reporter gene might be better. Previous study has shown that 547 or 729 genes out of total 31000 genes were significantly different between acutely isolated-astrocytes from P1 or P7 brain and cultured astrocytes, respectively (Foo et al., 2012, Neuron). Thus, although a part of genes may be affected by cultivation, we think that it is still possible to evaluate effect of CD38-deletion on gene expression of astrocytes. The RNA-seq analysis and pathway analysis and gene list of up- and downregulated genes are shown in Figure 7 and table EV2 and 3.

As we responded in the Comment#2 of the Reviewer1, we performed MACS-based isolation of astrocytes from CD38 AS-cKO^{P10} mice at P20. We confirmed that CD38 was specifically deleted in astrocytes (Fig EV1).

Comment#7

<u>Comparison between astrocyte-specific cKO and global KO</u>: Considering the abundant expression of CD38 in astrocytes compared to other cell types in the brain, I am wondering whether the comparison between the current astrocyte-specific CD38 cKO and the previous constitutive CD38 KO mice would provide a different phenotype with respect to its importance in synaptic function in neural circuits that mediate social behaviors in various brain regions. The authors note the importance of CA1, CA2, and NAC in social memory, but they only assessed synapses in mPFC. Multiple studies, including one from the authors, have reported that constitutive CD38 KO mice exhibit impaired social behaviors. Expanding beyond what is already known would require better spatial and temporal regulation of CD38 expression than presented here.

To compare social behavior between constitutive CD38 KO and CD38 AS-cKO^{P10} mice, we further performed 3 chamber social behavior test of WT and constitutive CD38 KO mice. In contrast to specific impairment of social memory in CD38 AS-cKO^{P10} mice, constitutive CD38 KO mice exhibited impaired not only social memory but also social preference (Fig EV3A–C). This difference is likely attributed to different oxytocin levels. In constitutive CD38 KO mice oxytocin level is half of WT mice (Jin et al., 2007), whereas its level was not changed in CD38 AS-cKO^{P10} mice. And reportedly, impaired social preference is recovered by oxytocin administration (Sala et al., 2013). On the other hand, the reduction of synapse number in the mPFC of constitutive CD38 KO mice (Appendix Fig S3D) was similar with those in CD38 AS-cKO^{P10} mice (Fig 3D). However, there is slight differences in the number of VGlut1 puncta (Appendix Fig S3B) and length of primary dendrites of pyramidal neurons in the mPFC (Appendix Fig S1B). Neuronal CD38 or astroglial CD38 at earlier than P10 may be involved in these difference. Because CD38 is expressed in neurons in the postnatal cortex and constitutive CD38 KO mice start to show impaired development of astrocytes and oligodendrocytes from P1 to P7 (Hattori *et al.*, 2017). We discussed the difference between the global KO and cKO in the Discussion section (line 397-411).

The hippocampus as well as the mPFC are critical regions for social memory (Okuyama *et al.*, 2016, Oliva *et al.*, 2020, Sun *et al*, 2020). To investigate whether astroglial CD38 is also involved in synaptogenesis in the hippocampus, we performed immunohistochemistry of VGlut1 and PSD95 in the CA1 and CA2 of the hippocampus of CD38 AS-cKO^{P10} mice. We found significantly decreased synapse number in the

stratum radiatum of the CA1 and CA2 region in CD38 AS-cKO^{P10} mice (Fig EV5A–D). Furthermore, we confirmed predominant CD38 expression in astrocytes in both the postnatal PFC and hippocampus by MACS-based astrocyte isolation (Fig EV1). Therefore, astroglial CD38 is involved in synaptogenesis at least these critical regions for social memory, which regulates social memory. We added the analysis in the hippocampus in Fig EV5A–D and the Results section (line 219-223), discussed a possible mechanism of the specific social memory impairment (line 376-396). Furthermore, we changed the title of our manuscript from "Astroglial CD38 regulates social memory and synapse formation through SPARCL1 in the medial prefrontal cortex" to "Astroglial CD38 regulates social memory and synapse formation in the developing brain".

Comment#8

<u>Rescue experiments:</u> The authors claim that reduced levels of Hevin secretion are responsible for reducing intracortical synapses in mPFC and the inability of their CD38 KO ACM to stimulate synapse formation. However, Hevin has primarily been linked to the formation of VGluT2+ synapses with only a transient effect on VGluT1+ synapses. Furthermore, Hevin's synaptogenic effect in astrocyte conditioned media is masked by its homolog Sparc. To claim that Hevin is responsible for reducing VGluT1+ synapses in mPFC the authors need to do a rescue experiment by expressing hevin in CD38 KO through AAVs brains or intracortical injections of recombinant Hevin.

To examine importance of SPARCL1 in astroglial CD38-mediated synapse formation, we performed in vivo recovery experiment using SPARCL1 recombinant protein. We injected recombinant SPARCL1 protein into Layer II/III of CD38 AS-cKO^{P10} mPFC at P15, then synapse number at P18 was examined by immunohistochemistry of VGlut1 and PSD95. Injection of SPARCL1 protein recovered synaptogenesis in the mPFC up to 87.9% ± 4.6 % of control mice (Fig 5C and D). We also found that treatment of SPARCL1 significantly recovered the decreased number of synapses in neurons cultured in CD38 KO-ACM up to 87.1% ± 0.09 of those of WT ACM (Fig 5A and B). Therefore, we concluded astroglial CD38 regulates synaptogenesis in the mPFC mainly through SPARCL1. We described these results in the Results section (line 283-292) and detailed method in "In vivo SPARCL1 injections" of the Materials and Methods section (line 691-704).

Comment#9

<u>Other synaptogenic factors</u>: The authors focus on Sparcl1/Hevin; however, other synaptogenic factors have been reported to affect VGluT1+ excitatory synapse formation and development directly. Notably, thrombospondins have been shown to regulate the formation of this specific synapse type through their receptor a2d1. The authors do not report any investigation into this family of factors despite their clear link to VGluT1+ synapse development.

As the reviewer commented, thrombospondin1 (TSP1) has been reported to regulate Vglut1 positive synapse formation (Eroglu et al., 2010, Cell). In our proteomics analysis, TSP1 level was not changed in CD38 KO ACM (122.1 ± 35.31 %; CD38 KO ACM/WT ACM) compared with that in WT ACM (Table EV1). We further examined amount of TSP1 and BDNF protein in CD38 KO ACM, their level was not changed in CD38 KO ACM (Figure 4B). In addition, expression of TSP1 in the developing brain is earlier than that of CD38 and SPARCL1. TSP1 expression is peaked at P8 and become undetectable already at P21 (Christopherson et al., 2005, Cell). Thus, we concluded thrombospondin1 is not involved in CD38-mediated synaptogenesis. We described these results in the Results (line 257-264) and Discussion section (line 421-426).

Comment#10

<u>Effect of CD38 cKO on astrocyte numbers</u>: The authors note that CD38 cKO alters GFAP expression; however, they also report a decrease in the number of GFAP+ and S100β+ cells without a change in NDRG2+ cells. The authors should address this discrepancy in astrocyte numbers with additional known markers such as Sox9.

To clarify whether astrocyte number is altered in CD38 AS-cKO^{P10} mice, we quantified Sox9 positive cells by immunohistochemistry as the reviewer recommended. Consistent with the number of NDRG2 positive astrocytes (Fig EV2 O and P), the number of Sox9 positive cells was not altered in the mPFC of CD38 AS-cKO^{P10} mice (Fig EV2 Q and R). Thus, we concluded deletion of astroglial CD38 affect some astrocyte specific protein expression such as GFAP, s100b and Cx43 without changing the number of the cells. These results are described in the Results section (line 143-145) and shown in Fig EV2.

Comment#11

<u>MBP quantification</u>: The authors previously reported changes in MBP expression

and oligodendrocyte maturation in the global CD38 KO animals. However, there is no quantification of the MBP staining in the cKO in supplementary figure 1. It would be important to verify that white matter structures developed properly in their cKO model, especially in mPFC.

Although we quantified MBP expression in the cortex of CD38 AS-cKO^{P10} mice, MBP expression was not changed in this cKO mice (Fig EV2 S). This result is inconsistent with our previous result using constitutive CD38 KO mice (Hattori et al., 2017, GLIA). In constitutive CD38 KO mice, expression of myelin protein such as MBP and CNP is already lower than that of WT at P7. We think that deletion of CD38 at earlier than P10 affects differentiation/maturation of oligodendrocytes. Thus we concluded that deletion of astroglial CD38 from P10 brain does not affect myelin formation. This result is described in the Results section (line 145-148) and shown in Fig EV2 S and T.

Minor Concerns:

1. SPARCL1 annotation should be Sparcl1.

SPARCL1 is often used in recent papers (Gan et al., 2020, J Neuroscience, Liu et al., 2022, Development), thus we keep SPARCL1 annotation in the revised manuscript. We added another name of SPARCL1 “Hevin” in the abstract and introduction section.

2. Avoid repetition of the same sentences in multiple places. E.g., The sentence- "Social behavior is essential for the health, survival, and reproduction of animals" is repeated both in the abstract and introduction.

We apologize for these repeated expression in the previous manuscript. We revised the whole Introduction section including this repeated expression as responded in the next comment.

3. The introduction needs to be thoroughly revised. In the first paragraph, a description of various studies(Fmr/Mecp2) which indicated the importance of synaptic function in neural circuits that mediate social behaviors in various brain regions could be presented later part of the introduction in a very concise manner since the article doesn't cover anything related to these genes. This part can be presented along with the narration of CD38, where authors described its importance

in social behavior. Introduce the importance of social behavior and their behavioral paradigm, especially what social memory is and what brain regions are important for it.

We thank the reviewer for this suggestion. We revised the whole Introduction section. In the first paragraph we mentioned roles and importance of social behavior, a role and critical brain regions of social memory and importance of neural circuit formation for social behavior. In the second paragraph, we mentioned the importance of astrocytes for neural circuit formation and cognitive behaviors. In the third paragraph, we introduced roles of CD38 for calcium signaling, social behaviors and astroglial development.

4. Introduction feels too short and abrupt.

We revised the Introduction section as the Reviewer#1 suggested above.

5. In Figures 2 and 3, Are the spine numbers/density/synapses affected in the p42 ctrl/CD38 AS-cKO group compared to the p10 ctrl/CD38 AS-cKO group?

To clarify whether synapse formation is impaired in CD38 AS-cKO^{P42} mice which did not show social memory deficit (Fig 1G-I), we quantified VGlut1, PSD95 and synapse densities in CD38 AS-cKO^{P42} mice. We found that any of Vglut1, PSD95 or synapse density was not altered in CD38 AS-cKO^{P42} mice. These results are described in the Results section (line 223-226) and shown in Appendix Fig S2.

6. In Figure 2; The authors should compare both the behavioral phenotype seen in two different tamoxifen injection/time points with the respective constitutive CD38 KO mice data.

To compare phenotype of social behavior between constitutive CD38 KO and CD38 AS-cKO^{P10} mice, we performed 3 chamber social behavior test of constitutive CD38 KO mice. CD38 AS-cKO^{P10} mice exhibited specific impairment of social memory, whereas constitutive CD38 KO mice exhibited impaired both social memory and social preference as previously reported (Kim et al., 2016, Mol Brain). This difference of social behavior is likely attributed to different oxytocin levels as described in the Comment#6. These results are shown in EV Fig 3, described in the Results section (line 164-167) and discussed in the Discussion section (line 397-404).

7. In Figures 3 and 4, the authors should analyze the spine numbers/density both in WT or CD38 KO ACM treated experiments and Sparcl1 KD/Sparcl1 treated rescue experiments?

We performed rescue experiment using SPARCL1 recombinant protein in WT or CD38 KO ACM treated neurons. SPARCL1 treatment significantly increased synapse number in CD38 KO ACM treated neurons and showed tendency to increase synapse number in WT ACM treated neurons (Fig 5A and B). These results are also described in the Results section (line 281-283).

8. The discussion section needs to be revised to reflect better the conclusions drawn from the data without overstatement.

We revised the Discussion section to accurately interpret the results in this study and clearly labelled speculation when we describe it.

Reviewer #2

SUMMARY

In their manuscript, Hattori et al., put forward evidence that the knock-out of CD38 expression in astrocytes at approximately post-natal day 10 (referred to as CD38 AS-cKO P10) leads to a specific deficit in social memory in adult mice, while other types of memory remain unaltered. Using immunohistochemistry (IHC), the authors found a reduced number of excitatory synapses in the medial prefrontal cortex (mPFC) of CD38 AS-cKO P10 mice. Switching to in vitro primary cell culture models, the authors identify the astrocyte secreted protein SPARCL1 as a relevant synaptogenic factor. Using pharmacological dissection of relevant signaling pathways, Hattori et al., propose that cADPR formation and calcium released from intracellular stores, is essential for SPARCL1 secretion from astrocytes. Finally, the authors analyzed the transcriptome of primary CD38 KO astrocytes using bulk mRNA sequencing, and found that genes related to calcium signaling were downregulated in these cells.

Comment#1

- Are the key conclusions convincing?

1) From a global perspective, the multiple lines of evidence provided by the authors strongly suggest that expression of CD38 in astrocytes is important for synaptogenesis in the mPFC of P10 mice, with ablation of CD38 and reduced synapse formation leading to social memory deficits at P70. However, the data concerning the role of astrocyte-secreted SPARCL1 is not particularly strong: further experiments are needed to support this claim (see below).

To confirm the importance of SPARCL1 for the CD38-mdiated synaptogenesis, we performed recovery experiments using SPARCL1 recombinant protein in vitro and in vivo (Fig 5). We confirmed and described the involvement of SPARCL1 in CD38-mediated synaptogenesis in the response to Comment #6 of the Reviewer 2 below.

Comment#2

- Are the claims preliminary or speculative?

1) As it stands, there is no proof that the claimed astrocyte-specific deletion of CD38 is actually astrocyte specific. This evidence is crucial: without it the reported effects could be due to non-specific CD38 knock-out in other CNS cells. In this respect, the Western Blot in Supplementary Figure 1A does not provide information on astrocyte-specific deletion, merely that CD38 was globally reduced in the mPFC. Interestingly, the authors have previously published data (Hattori et al., 2017, 10.1002/glia.23139) showing that CD38 expression is mostly astrocyte-specific, peaking at p14, which coincides with the peak period of synaptogenesis. The degree of CD38 heterogeneity is also an issue that I think the authors need to consider. Do they information on this? Is CD38 expressed in every astrocyte of the CNS, or are there some astrocytes that are CD38 negative at P14? Is the mPFC a region specifically enriched in CD38 positive astrocytes and does this explain the observed behavioral deficit? I think if this is known, the authors should mention it in the "Introduction" or "Discussion". If this is not known, maybe the authors could provide data addressing the issue.

To confirm astrocyte-specific deletion of CD38 in CD38 AS-cKO^{P10}, we isolated astrocyte from the PFC and hippocampus of CD38 AS-cKO^{P10} mice at P20 by magnetic-activated cell sorting (MACS) with the astrocyte surface antigen ACSA2 as previously performed (Holt *et al*, 2019; Kantzer *et al*, 2017; Ohlig *et al*, 2021). We

confirmed successful isolation of astrocytes and found that 86.5% (PFC; Fig EV1A) and 88.2% (Hippocampus; Fig EV1F) of significant reduction of *Cd38* mRNA expression in astrocyte fraction of CD38 AS-cKO^{P10} mice without changing *Cd38* expression in the astrocyte-negative fraction. These results indicate astrocyte-specific deletion of CD38 in CD38 AS-cKO^{P10} mice and described in the Results section (line 122-132).

To examine heterogeneity of CD38 expression in astrocytes, we performed immunohistochemistry or in situ hybridization of CD38 in CD38 AS-cKO^{P10} mice. Although we tested three anti-CD38 antibodies (AF4947, R&D systems; 102702, Biolegend; NBP2-25250SS, Novus Biologicals) for immunohistochemistry, we could not observe clear difference of CD38 expression between WT and CD38 KO mice by using any of these antibodies. Furthermore, we performed in situ hybridization–immunohistochemistry to detect *Cd38* mRNA in astrocytes. However, we found it also difficult to determine heterogeneity of *Cd38* mRNA expression by this method. As described above, we performed MACS-based astrocyte isolation from CD38 AS-cKO^{P10} mice at P20. This method is not able to clarify the heterogeneity. However, in the astrocyte fraction *Cd38* mRNA expression was 86.5% decreased in the cKO (Fig EV1A) and *s100b* mRNA level is more than ten times than that in the negative fraction (Fig EV1 B). Furthermore, we found most of the astrocytes are s100β-positive protoplasmic astrocytes in the mPFC (Fig EV2 M and N). Thus, CD38 is expressed at least in protoplasmic astrocytes in the mPFC.

Previous studies have shown that not only mPFC but also CA1 and CA2 of the hippocampus are critical regions for social memory (Hitti & Siegelbaum, 2014; Okuyama *et al.*, 2016; Xing *et al.*, 2021). We found that the number of synapses was also significantly decreased in the the CA1 and CA2 region of the hippocampus of CD38 AS-cKO^{P10} mice (Fig EV5A–D). Furthermore, the MACS-based isolation of astrocytes revealed predominant expression of CD38 in astrocytes in both the mPFC and hippocampus (Fig 1A and F). Taken together, astroglial CD38 promotes synapse formation in these critical regions for social memory. In our study the reduction of synapse number was 82.2%, 78.9% and 74.3% in the mPFC, CA1 and CA2, respectively. The synaptic reduction in the mPFC of CD38 AS-cKO^{P10} mice seems insufficient to cause behavioral impairment compared with chemogenetic inhibition of neurons in the previous study (Xing *et al.*, 2021). The mild synaptic reduction in these critical regions for social memory likely caused specific impairment of social memory in CD38 AS-cKO^{P10} mice. We described this possible mechanism in the Discussion section (line 376-396). We also changed the title of our manuscript from “Astroglial CD38 regulates social memory and synapse formation through SPARCL1 in the medial

prefrontal cortex" to "Astroglial CD38 regulates social memory and synapse formation in the developing brain".

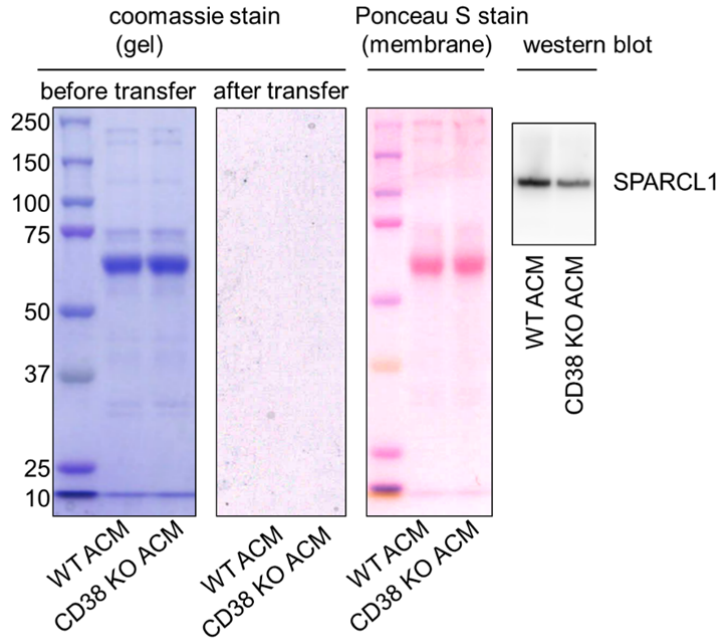
Comment#3

2) I think the authors should take more caution in claiming that SPARCL1 is the main factor secreted through the CD38 signaling pathway and responsible for increased synaptogenesis. This is for several reasons, all centered on data displayed in Figure 4 and Supplementary Figure 6:

a) Western Blot (WB) data: The "Materials and Methods" section for WB does not indicate how protein loading and transfer efficiency were controlled for. Normalizing to β-Actin levels is an acceptable way to control for loading and transfer efficiency when using cell lysates. However, in the absence of such an abundant structural protein in conditioned media it is unclear how loading and transfer was controlled for under these conditions. Do the authors normalized the CD 38 KO AS ACM data by expressing protein levels relative to those from WT AS ACM? Is BDNF being used as a control, based on proteomics data? If so, why is proteomics data not given in the manuscript and why is this control not shown for all ACM blots? I realize that (quantitative) blotting using ACM is difficult, but I am also not convinced that the methodology used is sufficiently rigorous. Simple steps to give confidence would be Coomassie staining of gels both before and after membrane transfer, to show that i) the total protein amount loaded was the same in each lane of the gel and ii) the transfer to the nitrocellulose membrane was complete. In addition, Ponceau S staining of the nitrocellulose membrane should also have been performed and displayed, to show (roughly) equal amounts of protein were transferred for each lane. In summary, the WB data quantification needs to be better controlled.

We thank the reviewer for this valuable suggestion. As the reviewer commented, amount of protein loading is critical for comparison of specific proteins such as SPARCL1 between WT and KO ACM. To normalize the results of western blot analysis of ACM, we performed Coomassie Brilliant Blue (CBB) staining of the gels in which equal amount of protein of each sample was loaded, and the density of the western blot was normalized by the density of CBB staining. By this normalization we confirmed the decreased amount of SPARCL1 in CD38 KO ACM (Fig 4A and B). We also normalized all the western blot analysis of ACM, added images of CBB staining and the normalized results in the Figure 4A and B, Figure 6B and C, and Appendix Figure S5A. As the

reviewer suggested, we confirmed complete transfer of proteins by CBB staining the gels after membrane transfer. We also performed Ponceau S staining of the membrane after transfer and confirmed equal amount of proteins were transferred as shown below. However, because of limitation of the space in the Figures, we showed only images of CBB stained gels in Figure 4A, 6B and Appendix Figure S5A without the images of CBB stained gels after transfer and Ponceau S staining of membranes. We described these methods in “Western blot analyses” of the Materials and Methods section (line 606-610).



Comment#5

The values of the Y axis in these graphs (and throughout the manuscript) are simply too small to be read properly. Finally, I want to highlight the general lack of precision regarding the nature of the replication unit (the "n"). For example, the legend of Figure4C-D states "n = 6", but we have no idea if these are 6 independent primary cultures originating from 6 mice, 6 independent cultures from the same mouse, 6 repeats of the Western Blot using the same sample etc. This issue is valid for the whole manuscript: in my opinion, the authors should be more much careful when it comes to these crucial elements of scientific reporting.

We reviewed all the graphs and images in Figures and revised appropriately them by

following the Figure Guidelines of EMBO Press. We apologize for inappropriate expression of number of experiments. We revised all these expressions such as "*n* = 6 independent culture per condition from 6 animals (Figure 4B, C) and "*n* = 4 animals per genotype (Figure 4H)" in the whole Figure Legends.

Comment#6

b) While the data hint at an important role of SPARCL1 in synapse formation, when the authors tested if ACM from CD38 KO astrocytes supplemented with exogenous SPARCL1 could rescue synapse formation, the effect was incomplete, with only a trend to an increase in synapse number (Figure 4J-K). Perhaps the authors simply forgot to indicate the statistical significance of differences between the experimental groups (Figure 4K)? However, if there really were no statistically significant differences observed, the authors should reduce the strength of their conclusions regarding SPARCL1. This protein may well be pro-synaptogenic but, as it stands, other factors could well be in play. Perhaps the authors should have tried higher concentrations of SPARCL1 to further boost synaptogenesis? In this respect, the SPARCL1 knockdown (KD) experiment in Supplementary Figure 6B-D is an important addition, but should be supplemented by rescue with an siRNA-resistant recombinant SPARCL1? If SPARCL1 is a major player in synaptogenesis, the prediction is that synapse numbers would be close to wild type levels with this approach.

To confirm the role of SPARCL1 for astroglial CD38-mediated synaptogenesis, we performed two recovery experiments using SPARCL1 recombinant protein. First, as described above in the response to Commnet#2 of the Reviewer2, we evaluated synapse number in neurons cultured with CD38 KO ACM by treatment of SPARCL1 protein. As the Reviewer1 requested to do this experiment with neurons without ACM as a negative control, we repeated and increased the number of this experiment to n = 40 to 55 cells per condition from 5 independent culture. Treatment of SPARCL1 significantly recovered the decreased number of synapses cultured in CD38 KO-ACM up to 87.1% ± 0.09 of those in WT ACM (Fig 5A and B). Second, we tested whether injection of SPARCL1 protein into the developing mPFC recovered the decreased synapse number in CD38 AS-cKO^{P10} mice as previously described (Risher et al., 2014, elife). We injected recombinant SPARCL1 protein into Layer II/III of CD38 AS-cKOP10 mPFC at P15, then synapse number at P18 was examined by immunohistochemistry of VGlut1 and PSD95. SPARCL1 injection significantly recovered the decreased excitatory synapse in CD38 AS-cKO^{P10} mice up to 87.9% ± 4.6 % of saline-injected cont^{P10} mice

(Fig 5C and D). Taken together, we concluded that CD38 promotes excitatory synapse formation mainly through SPARCL1 from astrocytes. These results are described in the Results (line 279-292) and Discussion section (line 414-421).

Comment#7

c) In my opinion, there are also issues with the data displayed in Figure 4H-I. The authors want to convince the reader that SPARCL1 is mostly an astrocytic protein using immunohistochemistry on mouse mPFC sections, co-labelled with antibodies against neuronal and astrocytic markers. In these panels, we are presented with images showing a few cells, in which it seems SPARCL1 is absent from NeuN positive cells, present in WT astrocytes and reduced in CD38 AS-cKO P10 astrocytes. However, the numbers of cell counted and lack of quantification severely impact on the strength of this conclusion. In my opinion, the authors should have quantified their IHC data by counting cells and establishing the ratios of SPARCL1 positive over NeuN or S100β positive cells, in both control and CD38 AS-cKO P10 animals. This experiment would provide critical information that the conditional gene targeting strategy is robust. The authors should also consider quantifying the intensity of the SPARCL1 signal in astrocytes. This is recommended as the image displayed in Figure 4I for the CD38 AS-cKO is problematic: are the authors really claiming that the reduction in SPARCL1 expression following cKO of CD38 in astrocytes is at best only partial? Is 11 days between the first tamoxifen injection and tissue fixation actually sufficient to allow for CD38 turnover? With low levels of protein turnover, the possibility exists that residual levels of CD38 are still sufficient to impact SPARCL1 levels. What would happen if there is a greater interval between tamoxifen administration and tissue recovery? Would levels of synaptogenesis be further reduced? Is this an issue of production versus secretion or a combination of factors?

To confirm decreased SPARCL1 expression in astrocytes of CD38 AS-cKO^{P10} mice, we quantified number of SPARCL1 positive astrocytes and neurons in the mPFC. We found that SPARCL1 is predominantly expressed in astrocytes and the number of SPARCL1 positive astrocytes was significantly decreased in CD38 AS-cKO^{P10} mice. The quantified data is shown in the Figure 4H and images were replaced representative images to show several SPARCL1 positive astrocytes and their magnified images (Figure 4G).

Comment#8

3) The heatmap (Figure 5E-F) is simply too small to interpret. The color choice is also not accessible for colorblind readers. The authors might consider displaying this heatmap in a separate figure. The authors should also provide a supplementary table where all the genes detected are listed along with their respective counts. Furthermore, it is surprising that the authors only found genes being downregulated in CD 38 KO astrocytes. Were they really no genes up-regulated? The authors might also want to indicate the genes belong to each of the ontological categories listed in Figure 5F. On p. 11, Figure 5E: The authors should indicate in the main text they performed bulk RNA-sequencing and not another type of RNA sequencing (like single cell RNA sequencing for instance). The authors indicate n = 2 but we have no indications of the nature of the replicate (also see earlier comments). Please amend.

We changed color and size of the heatmap and moved it to Figure 7A. The down- and upregulated pathways are also shown in Figure 7B and C. The list of down- and upregulated gene in CD38 KO astrocytes are shown Table EV2.

We apologize for insufficient information regarding RNA-seq. We described as follows in the Results section "we performed bulk RNA-sequencing (RNA-seq) on cultured astrocytes from WT and CD38 KO mice at P1 (n = 2 independent cultures per genotype)" (line 310-312).

Comment#9

- Are additional experiments necessary?

I think supplementary experiments are essential to support the claims of the paper. Most are described in the section above, but to summarize:

1) Show data to prove that the CD38 AS-cKOP10 model is astrocyte-specific and leads to a total loss of CD38 in these cells.

We responded in Comment#2 of the Reviewer2.

2) WB data: The issue of protein loading and transfer efficiency should be dealt with. Quantifications should be revisited.

We responded in Comment#3 of the Reviewer2.

3) The authors should quantitatively analyze the different IHC performed in Figure 4H-I.

We responded in Comment#7 of the Reviewer2.

4) The authors should provide more information on their RNA sequencing data: list of genes detected with their FPKM values etc. The authors should display the RNA sequencing data in a separate figure, allowing the heatmap to be enlarged.

We responded in Comment#8 of the Reviewer2.

5) LC-MS/MS data: the authors should provide the list of all the proteins they identified in their LC-MS/MS experiment. As a supplementary table for instance?

We thank the reviewer for this suggestion. We provided the list of the identified proteins in Table EV1.

Comment#10

- Data and methods presentation

Methods:

The authors need to work on this aspect of the manuscript. Most of the important details are already described, but some crucial ones are missing, while the phrasing used to describe methods is sometimes misleading. I will give some examples here, but this is not an exhaustive list. The fact that the manuscript is riddled with small mistakes, inconsistencies and/or oversights makes it difficult to read and creates a negative impression. The whole manuscript would benefit from a thorough proof-reading, preferably by a native speaker.

We revised the whole Materials and Methods section by describing sufficient detail that other researchers can repeat our experiments. And proof-reading of the whole manuscript also has done by a native speaker.

Comment#11

1) in the "Immunohistochemistry and Synaptic Puncta Analysis" section on p. 21-22, we have no indication of which antibodies against "GFAP, NDRG2, VGlut1, PSD95, S100β, NenN(?) and SPARCL1" were used. It is standard practice to indicate the company, product number and lot number. The authors must also indicate the dilution at which they use these antibodies. On p.22, the authors write the cells were

incubated with "Alexa- or Cy3-conjugated secondary antibodies". The excitation wavelengths of the Alexa dyes used need to be given.

We described catalog number, company and dilution of all these primary, secondary antibodies, and all the materials in the Materials and Methods (line 619-628).

Comment#12

2) The authors need to provide more details on the microscope they used. Merely writing "using a 63× lens on a fluorescence microscope" (p.23) is insufficient.

We added information of the microscopy as" ~ were imaged with a 63×/1.20 W HC PL APO CORR CS2 objective on a Dragonfly confocal laser scanning microscope." in the Materials and Methods section (line 636-638).

Comment#13

3) In the "LC-MS/MS" method the authors wrote: "Briefly, these proteins were reduced, alkylated, and digested by trypsin". I think that in the reduction and alkylation steps, chemicals other than trypsin were actually used. This sentence should be modified to reflect this.

We revised this description as follows, "these proteins were reduced, by tris(2-carboxyethyl)phosphine (TCEP), alkylated by iodoacetamide (IAA), and digested by trypsin." (line 673-675).

Comment#14

4) p.19: "uM" is written when the authors very likely mean "μM". Please check the whole manuscript for repeat examples. I know this is often lab "short-hand", but it should be avoided in scientific publications.

We checked and corrected all these errors throughout the manuscript.

Comment#15

5) The authors should be careful when describing their data to always indicate whether they referring to experiments performed using cultured astrocytes or not. As it stands, the text is confusing: for instance, when describing RNA-sequencing data in Figure 5, the main text appears to indicate that these astrocytes were acutely isolated

from adult mice, when in fact they were obtained from primary cultures. Given concerns in the literature about potential differences between acutely isolated and cultured astrocytes (Foo et al., Neuron, 2011), this is essential.

As the reviewers commented, reportedly there is difference of gene profile between acutely isolated from P1 brain and cultured astrocytes (Foo et al., Neuron, 2011). To clearly indicate the experimental condition of our RNA-seq analysis, we described as follows "we performed bulk RNA-sequencing (RNA-seq) on cultured astrocytes from WT and CD38 KO pups (n = 2 independent cultures per genotype)." in the Results section (line 310-312).

Comment#16

1) Almost all Y axis labels are too small. The authors should comply to the basic journal requirements in terms of font sizes. Some axes do not end on a tick (e.g. Figure 3R). This is not dramatic, but should be corrected. Globally, the authors need to display bigger bar plots - most of them are extremely hard to read. Labeling should also be checked: Figure 4K, the Y axis label indicates values displayed are in %, when I think the axis graduation displays ratio values. Some of the IHC pictures are also too small to be easily interpreted.

We checked and revised font, graphs and pictures in all the figures as the EMBO Press Figure Guideline required.

Comment#17

2) The heatmap in Figure 5E is impossible to read and, as such, has little or no value for the manuscript.

As described in the response to Comment #8 of the Reviewer 2, we changed color and size of the heatmap and moved it to Figure 7A. The down- and upregulated pathways are also shown in Figure 7B and C, respectively. The down- and upregulated gene lists are shown Table EV2.

Comment#18

3) Scale bars: where is the scale bar in Figure 2A? Figure 3A-H: Is the scale bar really representing 10 millimeters? Supplementary Figure 3A: scale bar is missing. Please check for similar issues throughout the manuscript.

We checked all the scale bars and put them appropriately in all the figures.

Comment#19

4) Figure Legends are problematic, and often contain incorrect or incomplete information. Examples include: Supplementary Figure 1: The description of panels J, L and N appears to be missing. Please also use the Greek letter beta and not 'b' for S100β. Supplementary Figure 5: I think the term "KO" is missing after CD 38 in the legend title. Figure 3: why state that nuclei were counterstained with DAPI in Figure 3P,Q, when this precision is not given for panels Figure 3A-H? Figure 3A-H: If the authors choose to explicitly state PSD95 is a post-synaptic marker, why not indicate that VGlut1 is a pre-synaptic marker? Same issue in Supplementary Figure 4.

We corrected these errors the reviewer commented above. Furthermore, we thoroughly revised the Figure legends of main figures, Expanded View and Appendix as the EMBO Journal Guideline required.

Comment#20

5) There are multiple instances of panels being wrongly referred to in the main text. On p.10, Figure 4H is referenced, when I think the authors mean Figure 4I; on p.10, Figure 4I-J are referred to when the authors clearly describe data found in Figure 4J-K. These types of mistakes are problematic and recur throughout the manuscript.

We carefully examined references throughout the manuscript and corrected errors including the reviewer pointed out.

Comment#21

As mentioned above, the exact nature of the replicates is often not stated, when the "n" number is indicated. The authors must correct this issue and give the information either at the appropriate point in the main text or in the figure legend.

We revised all these expressions regarding number of experiments such as "n = 6 independent culture per condition from 6 animals (Figure 4B, C) in the whole Figure Legends.

Comment#22

The authors should also be more consistent in the way they indicate which statistical tests were performed. This should also be indicated either at the appropriate point in the main text or in the figure legend. Furthermore, care should be taken to ensure statistics are presented in an appropriate manner: at the end of legend for Figure 4, it is indicated #p < 0.05 vs. CD38 KO ACM. This hashtag symbol is completely absent from the figure. In Figure 4F-G, the lack of statistical symbols seems to indicate no statistical tests were performed on these data, when the legend covering these panels states "*p < 0.05 versus P70", indicating some tests were done. We cannot interpret this panel without knowing which comparisons were done exactly and which were significant.

We checked all the statistics and their presentation in the whole manuscript and revised if necessary as the Journal's Guideline required. We apologize for incorrect description of statistics in Fig 4. We indicated the statistical test used for these figures in the Figure legend of Fig 4E and F and added asterisks in these figures.

Comment#23

In the "Materials and Methods", the authors give no indication that the assumptions of the statistical test they used were met (normality of data distribution for t-tests, homogeneity of variances for ANOVA...). This needs to be checked, and if not met, appropriate non-parametric tests should be used instead.

We thank the reviewer for this comment. We first determined whether data were normally distributed or not. Then, for parametric data we used e unpaired Student's t-test, one-way ANOVA, or two-way ANOVA and appropriate post hoc analyses. For non-parametric data, the Mann-Whitney *U*-test (Fig 3D and Fig EV5F) or the Kruskal-Wallis test with *post hoc* Dunn's test (Fig 5D) was used. We described these statistical methods in the Materials and Methods section (line 843-849) and Figure legends.

MINOR COMMENTS:

- Clarity and accuracy of text and figures

There are issues with the clarity and accuracy of text and figures - which are described above. The text is also often problematic in its phrasing and other, more fundamental aspects. For instance, the authors spent a considerable amount of time

speaking about the role of oxytocin, when they only performed one measurement of oxytocin levels in mice.

We thank the reviewers for all the valuable comments. We thoroughly revised thoroughly our text and figures to clearly and accurately describe our methods, results and their interpretation as described above.

We reduced the description regarding oxytocin in the Discussion section. We only mentioned involvement of oxytocin to compare behavior phenotypes between constitutive CD38 KO and our cKO mice (line 399-404).

- Suggestions to improve the presentation of data and conclusions?
All my suggestions to improve the presentation of data can found in previous sections. As for improving the authors presentation of their conclusions, the authors should make a considerable re-drafting effort, particularly for the "Discussion", which lacks clarity in how supporting arguments are built and presented. For example, on p.13, I am confused with the argument made by the authors. Their data are focused on synapses onto pyramidal neurons of the mPFC, but here the discussion states that the behavioral phenotype they observed in CD38 AS-cKOP10 might be explained by a lack of mPFC neurons synapsing onto neurons in the Nucleus Accumbens (assuming that "NAc" really refers to this brain region, as the definition is missing from the text). I think the authors should make it clear if this is their interpretation of their own result, which essentially renders their focus on mPFC pointless, or a speculation on possible other mechanisms that could also explain their behavioral results. Personally, given the data shown, I believe the authors should focus on explaining how their data in mPFC might explain the behavioral output observed. The authors could also provide perspectives on how the hypothesis laid down in this paragraph would be tested. When the authors write on p.14 "We identified SPARCL1 as a potential molecule for synapse formation in cortical neurons" why use the word "potential"? Does this mean the authors consider their data on SPARCL1 (one of the key messages of the paper) invalid? If the authors themselves think the role of SPARCKL1 is ambiguous based on their own data, they should perform further experiments. P. 13, the authors write: "Moreover, many studies have shown that astrocyte-specific molecules, including extracellular molecules such as IL-6, are involved in memory function"; Interleukin 6 (Il-6, abbreviation not defined in the manuscript) is definitely not an astrocyte-specific molecule (see, for example, Erta et al., 2021 10.7150/ijbs.4679).

We revised the discussion section to accurately interpret the results in this study and clearly labelled speculation when we describe it.

As we responded in the Comment#2 of the Reviewer2, previous studies have shown involvement of mPFC and CA1 and CA2 of the hippocampus in social memory (Hitti & Siegelbaum, 2014; Okuyama *et al.*, 2016; Xing *et al.*, 2021). Therefore, we further examined synapse formation in the CA1 and CA2 in CD38 AS-cKO^{P10} mice. Synapse number in the mPFC, CA1 and CA2 of CD38 AS-cKO^{P10} mice was significantly reduced to 82.2%, 78.9% and 74.3% of those in control mice, respectively (Fig 3D and Fig EV5). This mild synaptic reduction in multiple critical areas for social memory likely account for the impaired social memory. We described the possible mechanism for the specific impairment of social memory and the limitation of our study in the Discussion section (line 376-396).

As described in the Comment#6 of the Reviewer2, by performing recovery experiments by SPARCL1 in vitro and in vivo, we concluded astroglial CD38-mdiated synapse formation mainly through SPARCL1. We described this in the Discussion section (line 414-421).

We deleted the description regarding Interleukin 6.

Reviewer #3

SUMMARY

Hattori et al. assessed the role of astrocytic CD38 by generating astrocyte-specific conditional CD38 knockout mice and discovered defects in social memory, synapse, and spine density in the mPFC. They further showed that conditioned media from CD38-deficient astrocytes are defective in promoting synapse formation. A known astrocyte-derived synapse promoting protein, Sparcl1, is reduced in the conditioned medium from CD38 KO astrocytes and pharmacological experiments suggest that CD38 and calcium signaling regulates Sparcl1 secretion by astrocytes.

Comment#1

1. It's unclear if experiments were conducted while the experimenters are blinded to the genotype of the mice. This is essential for behavior tests.

We apologize for lack of information regarding behavioral tests. We performed all the

behavior tests in blinded manner. This is mentioned in the Materials and Methods section (line 503-504).

Comment#2

2. Hippocampus is also important for memory formation. Do synapse and spine densities change in the hippocampus?

As the reviewer commented, the hippocampus as well as the mPFC are critical brain region for social memory (Okuyama *et al.*, 2016, Oliva *et al.*, 2020, Sun *et al*, 2020). To investigate whether astroglial CD38 affects also affect synapse formation in the hippocampus, we performed immunohistochemistry of VGlut1 and PSD95 in the CA1 and CA2 of the hippocampus of CD38 AS-cKO^{P10}. We found that the number of synapses was significantly decreased in the stratum radiatum of the CA1 and CA2 region in CD38 AS-cKO^{P10} mice (Fig EV5A–D). Furthermore, we found predominant CD38 expression in astrocytes of the postnatal PFC and hippocampus by MACS-based astrocyte isolation (Fig EV1). Taken together, astroglial CD38 promotes synapse formation in both the mPFC and hippocampus which are critical region for social memory. We described these results in the Results section (line 219-223) and possible mechanism for specific impairment of social memory in CD38 AS-cKO^{P10} mice in the Discussion section (line 376-396). The title of our manuscript also changed to "Astroglial CD38 regulates social memory and synapse formation in the developing brain" from "Astroglial CD38 regulates social memory and synapse formation through SPARCL1 in the medial prefrontal cortex".

Comment#3

3. The proposed model of CD38 inducing Ryr3-mediated calcium release from internal stores is interesting. However, the Barres database showed that Ryr3 is not expressed by mouse astrocytes. Could the authors demonstrate the presence of Ryr3? That's a key link in their model that hasn't been demonstrated to operate in astrocytes.

A previous study suggests RYR1, 2 and 3 expression in astrocytes in the hippocampus and striatum by RNA-seq analysis on FACS-isolated astrocytes (Data S1; Chai et al., 2017, Neuron). We further examined expression of RYRs mRNA level in the astrocytes isolated from the PFC of ctrl^{P10} or CD38 AS-cKO^{P10} mice at P20 by qRT-PCR.

Although RYR1 and 3 were undetectable in these cells, RYR2 was expressed slightly lower level in ACSA2-positive astrocytes than those in the ACSA2 negative cells (Fig 4E). This result suggests at least RYR2 expressed in astrocytes in the developing PFC. This result is described in the Results section (line304-310) and shown in Fig 4E.

Comment#4

4. The authors demonstrated reduced synapse and spine density in mPFC. Interestingly, a battery of behavior tests showed no defect, except for the social memory test. Reducing synapses in mPFC should affect a range of behaviors. Why that is not the case here?

As the reviewer commented, the mPFC plays an important role in higher brain function, including cognition, motivation and emotion by connecting with other brain regions (Bolkan et al., 2017, Nat Neurosci, Burgos-Robles et al., 2017, Nat neurosci). In our study synapse number in the mPFC of CD38 AS-cKO^{P10} mice was reduced to 82.2% of those in control mice (Fig 3D). The synaptic reduction in CD38 AS-cKO^{P10} mice seems insufficient to cause behavioral impairment compared with chemogenetic inhibition of neurons used in the previous studies above. We also found 78.9% and 74.3% of reduced synapse number in the hippocampal CA1 and CA2 of the cKO, respectively (Fig EV5). The mild synaptic reduction in critical areas for social memory might account for the specific impairment of the behavior. However, deletion of astroglial CD38 may affect other memory functions. Because the hippocampus is a central region for coding of spatial, temporal and episodic memory, especially CA2 is required for social memory formation as well as encoding spatial memory (Kay *et al*, 2016). Moreover, constitutive CD38 KO mice shows memory deficit in fear conditioning test and Morris water maze test (Kim *et al.*, 2016; Martucci *et al.*, 2019). We described the possible mechanism for the specific impairment of social memory and the involvement of astroglial CD38 for the other memory functions in the Discussion section (line 376-396).

Comment#5

5. The authors only tested very short-term memory (30 minutes delay). Does CD38 regulate long-term memory? It would be important to know but I realize that a single paper cannot address all questions and therefore do not think addressing this point is a prerequisite for publication.

We thank the reviewer for this important comment. In the present study we only

examined 30 minutes of short-term social memory (Fig 1). The social memory deficit in CD38 AS-cKO^{P10} mice is likely attributed to impaired synapse formation in the mPFC and hippocampus. Synapse impairment such as decreased synapse number and morphological change can cause short- and long-term memory deficit as shown previously (Gao et al., 2015, Front Mol Neurosci.). Therefore, it is possible that CD38 AS-cKO^{P10} mice exhibit long-term social memory deficit for hours and days. We would like to examine roles of astroglial CD38 for long-term social memory and other types of memory in future study. We discussed these possible functions of astroglial CD38 in the Discussion section (line 390-396).

Minor Comments

1. Fig. 2F, multiple comparison adjustment is needed.

We apologize for lack of multiple comparison test in this analysis. We applied it to Fig 2F. and other statistical analyses if necessary. We added description regarding multiple comparison test in "Statistical analyses" in the Materials and Methods section (line 845-849) and Figure legends.

2. Fig. 3A, scale bar is 10 micrometers, not millimeters.

We corrected this error and added scale bars appropriately throughout Figures.

3. Fig. 4C, D, it is unclear if the quantification is normalized to actin loading control. BDNF levels appear lower in KO, though not significantly different, raising the question of whether an equal amount of samples was loaded.

As all the reviewers commented, amount of protein loading is critical for comparison of specific proteins such as SPARCL1 between WT and KO ACM. To normalize the results of western blotting of ACM, we performed Coomassie Brilliant Blue (CBB) staining of the gels where the protein samples were loaded, and the density of the western blot was normalized by those of CBB staining. By performing this normalization, we confirmed the decreased SPARCL1 in ACM by deletion of astroglial CD38 (Fig 4A and B), blocking of cADPR/calcium signaling (Fig 6B and C) and knockdown of astroglial SPARCL1 (Appendix Figure S5A). We described these procedure in "Western blot analyses" of the Materials and Methods section (line 606-610) and added images of CBB staining and the normalized results in the Figure 4A

and B, Figure 6B and C, and Appendix Figure S5A.

4. Need to validate whether CD38 levels are reduced in P42-46-injected adult knockout before concluding that CD38 is required only during development

We examined CD38 expression in CD38 AS-cKO^{P42} mice. We confirmed CD38 protein is significantly decreased in the mPFC of these mice at P70. This result is shown in Fig EV2 I and J and described in the Results section (line 171-172).

Dear Tsuyoshi,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the original referees. As you can see from the comments below, the referees find the analysis improved.

Referee #1 would like to see some further analysis including rescue analysis and looking at astrocyte morphology. I have discussed these issues further with referees #2 and 3 and while they both agree that such analysis would be insightful, they also find that this is a major undertaking and not needed for publication here. If you have data on hand to address these issues then by all means include it, but if not then we can move forward with the manuscript in the absence of such data.

I would however like to ask you to carefully re-write the manuscript and take a more nuanced approach. I would suggest to focus emphasise that 1) astrocyte-specific deletion of CD38 in development leads to defects in social behavior associated with synapse/circuit assembly issues 2) that one contributing factor is decreased synaptogenesis which can be explained by reduced release of pro-synaptogenic factors, of which Hevin is one; 3) a better discussion of the caveats of the study - limited mass-spec coverage, likely impact of other pro-synaptogenic factors etc.

I would also like to ask you to respond referee #3's concerns as well. Let me know if we need to discuss anything further happy to do so via email or a video call.

When you submit the revised manuscript will you also take care of the following points:

- COI needs to be renamed to "DISCLOSURE AND COMPETING INTERESTS STATEMENT"

- Please remove the Authors Contributions from the manuscript. The 'Author Contributions' section is replaced by the CRediT contributor roles taxonomy to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y')

-Please check figure callous for: Figure 3H, 6E, EV2B, EV2F, EV2G.

- Table EV1-EV3 should be renamed to Dataset EV1-EV3, uploaded as individual files with legends uploaded as separate tab in each Excel file. Please also correct corresponding callouts

- Page numbers are missing in the appendix for the ToC

- Source data files need to be reorganized and zipped to one file/folder per figure. For EV and/or appendix figures, ZIP together all source data.

- Please upload a synopsis text. We need a summary statement plus 3-5 bullet points describing the key findings of the MS.

- We also need a synopsis image => 550 wide by [200-400]
Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

- legend for Table 1 should be removed from manuscript file, as it is in the file Table 1

- For the data availability section please make sure that the RNA-seq data is available and open. Also remove the sentence: The data supporting the findings are available from the corresponding author (TH) upon request.

- Please double check the figures and that you have uploaded high-quality figures.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

This reviewer believes the authors adequately addressed most of the previous comments and is excited by the overall findings of the work presented. However, two major concerns were not addressed in work shown. This reviewer does not feel the current work is ready for publication without addressing these two concerns.

Major Concern 1:
The first major concern is that the authors have not conducted a rescue experiment in their social memory task in their P10 CD38 cKO mice. The authors provide sufficient data to support the claim that CD38 cKO impairs Hevin secretion in astrocytes and that Hevin is sufficient to rescue synaptic phenotypes found in the CD38 cKO in vivo. However, the authors did not test the sufficiency of Hevin administered in early life to rescue the social memory deficit seen in the P10 CD38 cKO or global CD38 KO. This is an essential experiment to show that Hevin is the critical molecule secreted by astrocytes during this developmental period that regulates the establishment of the social memory circuit the authors are investigating.

This reviewer believes that because the mass spectrometry data has limited coverage of the secreted astrocyte proteome, proteins involved in the researcher's behavioral task may still be missed. For this reason, this reviewer recommends that the researchers administer Hevin either by overexpression or direct injection of the purified protein, just as they did in their synapse rescue experiment into control and CD38 cKO mice. By testing this cohort later using their social memory task, the authors could then show that despite the limited coverage of their mass spectrometry data, they still identified an astrocyte-derived factor sufficient to allow for proper social memory circuit function.

Major Concern 2:
The second major concern is that the authors limited their investigation to astrocyte-secreted factors. This reviewer believes that the authors provided sufficient data to support the claim that astrocyte secretion of Hevin is impaired in their CD38 cKO. However, the data presented also suggests that astrocytes are broadly dysregulated in their CD38 cKO, given that they find decreased S100b and GFAP with no changes in Sox9 or NDRG2 cell numbers. A previous concern from this reviewer was that astrocyte morphology was not tested in the CD38 cKO model.

If the authors performed the behavioral rescue experiment using Hevin in the CD38 cKO mice and found this was sufficient to recover normal social memory, then this reviewer feels that further investigation into astrocyte morphology would be interesting but not essential. However, without the rescue data, there is no way to determine if the synaptic phenotypes observed and the behavioral phenotypes observed are linked and dependent on the astrocyte secretion of Hevin. Previously published work has shown that astrocyte morphology depends on interaction with neurons and that impaired astrocyte morphology impairs synapse development. Therefore, investigating astrocyte morphology in the CD38 cKO model would be informative, especially if Hevin cannot rescue social memory.

This experiment is also within the technical capabilities of the lab, given that it could be accomplished by crossing their Glast-creERT2/CD38 floxed mice with a cre-dependent reporter line (such as Ai14) and providing a low dose of tamoxifen. Alternatively, the investigators could use previously published methods to sparsely transduce astrocytes with a cre virus + fluorescent protein under the GFAP promoter or through electroporation using a GFAP cre-plasmid + fluorescent protein.

Minor concerns:
Other minor problems with the work presented are:

1. While the investigators conducted RNA sequencing on cultured astrocytes from control and CD38 KO mice, this reviewer feels that the investigators might be confounding their effects because of the presence of serum in their astrocyte cultures. A cleaner experiment would be to cross their cKO line to a Cre-reporter line and FAC sort astrocytes for RNA sequencing.
2. The synapse images would benefit from changing to green and magenta (instead of red and green) to accommodate color-blind readers.
3. The representative image for Figure 3 cKO PSD95 puncta does not show a reduction in PSD95 puncta. Perhaps a better representative image could be selected instead.

Referee #2:

The authors have adequately addressed all my concerns. The manuscript is an important contribution to the field. I recommend publication of this manuscript.

Referee #3:

Astroglial CD38 regulates social memory and synapse formation in the developing brain
Hattori et al.
In this manuscript, Hattori et al. put forward evidence that CD38 knock out specifically in astrocytes leads to a deficit in social memory in adult mice, while other types of memory remain unaffected. This effect is present only if CD38 is knocked out around post-natal day 10 (CD38 AS-cKO P10), since KO induction at later age has no (overt) effects. Using Golgi stainings and immunohistochemistry, the authors found a reduced number of mature excitatory synapses in the medial prefrontal cortex (mPFC) and hippocampus of CD38 AS-cKO P10 mice. Switching to astrocyte-neuron co-culture models (in which the cells remain physically separated), the authors obtain evidence that the presence of CD38 is essential for secretion of a pro-synaptogenic factor. Using proteomics on astrocyte conditioned media (ACM) from CD38 KO and wild type (WT) primary astrocytes, they identify a putative factor, SPARCL1 (Hevin), which has been previously implicated in glutamatergic synaptogenesis. Crucially, SPARCL1 expression is downregulated in both isolated CD 38 KO astrocytes and the intact mPFC of CD38 AS-cKO P10. Interestingly, exogenous SPARCL1 can partially rescue the loss of synapses observed in both the *in vitro* and *in vivo* models used. Applying pharmacological tools to their *in vitro* model, the authors further indicate that the CD38 signaling pathway, linked to downstream cADPR formation and calcium release from intracellular stores, is essential for SPARCL1 secretion from astrocytes. Finally, the authors analyzed the transcriptome of isolated CD38 KO astrocytes using bulk mRNA sequencing, and found that genes associated with calcium signaling pathways were downregulated in these cells, while inflammatory pathways genes were upregulated.

This is a revised manuscript of a version originally submitted via the Review Commons system. The authors have answered my main concerns, notably by demonstrating the astrocyte specificity of their transgenic mouse model, and providing more data on how the normalization of their western blot experiments was performed. The extension of some their conclusions to the hippocampus is interesting and increases the significance of the manuscript. While SPARCL1 has previously been implicated in synaptogenesis, to the best of my knowledge, this is the first time SPARCL1 and the CD38 signaling pathway have been linked, with astrocyte-specific CD38 ablation resulting in a specific functional deficit. While the experimental data has improved considerably, the largely rewritten 'Discussion' has some substantial issues which need to be responded to. If these are suitably addressed, alongside the other considerations listed below, then I would support publication in EMBO J, as the manuscript would be of interest both to the neuroscience and cell biology communities.

Major Comments:
(i) The data shown in Figure 4D, E and F is unconvincing. The blot data (Figure 4D) seems to show marked reduction in both SPARCL1 and CD38 expression at P70. However, this is not apparent in the quantification. I realize the data (Figure 4E, F) contains some low values, but perhaps a more representative blot would work better at this point. Likewise, the immunohistochemistry panels (Figure 4G and H) are also not particularly convincing.
(ii) In general, I felt the 'Discussion' was poorly written. In my opinion, it contained several factual inaccuracies, as 2APB is an IP_3 antagonist, while Mondal and colleagues demonstrated a primary role for fibromodulin in filamentous actin stress fibre formation and not extracellular matrix assembly. It is also unclear to me how the authors can rule out a role for TSP1 in glutamatergic synapse assembly based on the data they present (which runs contrary to the large body of published studies). Comparison of SPARCL1 levels to other studies (Risher et al., 2014) only holds if exactly the same protocols are used. Given the small number of proteins detected in the ACM analysis, I would have thought a discussion on the limitation of their proteomic analysis would have been valid. Furthermore, I was disappointed at the lack of any attempt to link/speculate on how the comparatively small decrease in SPARCL1 secretion and synapse number could relate to a specific behavior. Finally, I believe that the reference to 'mitochondrial transfer to neurons in ischemic brains' was outside the scope of this study and, therefore, superfluous. Overall, it is my opinion that the 'Discussion' would benefit from some critical editing, preferably to include proof-reading by a native English speaker.
(iii) Finally, based on all the data, can the authors justify the manuscript title 'Astroglial CD38 regulates social memory and synapse formation in the developing brain'. Wasn't social memory tested in adult mice?

Minor comments:
In general, the layout of the text and figures has clearly improved. Nonetheless, small issues remain, such as axis labels being at variable distances from the axis (e.g. Fig. 6C vs. 6D), or the value "-1" in Fig. 7A being above the scale when other values are below. These small issues must be corrected throughout the manuscript before publication.

Specific comments on science:
(i) Proof of specific CD38 KO in astrocytes: Page 6, line 132 and Figure EV2: If I understand correctly, Western Blots were performed with whole brain lysates, which is presumably why a residual level of CD38 is detected. In this instance, Western Blots performed using ACSA2-MACS purified astrocytes (as in Figure EV1), would be much more convincing for the story,

particularly if they demonstrated complete protein loss at 11 days post-tamoxifen injection, as I suspect they will do.
(ii) Figure EV2A: It is unclear to this reviewer, why we are shown what seems to be three replicates per genotype in the blot image, while the quantification was done on four animals. Are these technical replicates? If yes, could the author state this in the figure legend? Also in the legend, the word 'analyses' in (A) should be 'analysis'.
(iii) Figure 3 (and other relevant figures): Why was VGLUT1 used as the sole pre-synaptic marker? Are VGLUT2 levels so low in mPFC and hippocampus? A specific effect on VGLUT1 synapse assembly would be of great interest to the community. Why do there appear to be double bands in Figure 3E?
(iv) Figure 4A source data: Why are the blots for TSP1 and beta-actin not included?
(v) Figure EV1: This is a most welcome addition. I would just indicate on the figure that the first row of bar plots comes from mPFC samples and the lower one from hippocampal samples.

Specific comments on statistics:
Personally, I have a small issue with the fact the authors are not clearly displaying, either in the manuscript or in a supplementary table, the complete results of their statistical tests. Yet, a number of conclusions are drawn from such 'non-significant' comparative tests. I think they should be reported, as the *p*-value of these results is important for the reader to interpret the data. A table might be the easiest way, to not overload the figures.

Some examples:
(i) Figure 1: The authors display 'within group' statistical comparisons, which are, in general, interesting and needed, but wouldn't 'between group' comparisons also be of interest to reinforce the authors conclusions? This would allow the authors to legitimately write on p.8, line 173: "In these mice (CD38 AS-cKOP42), all social behaviors, sociability, social preference, and social memory were similar to those of ctrlP42 mice (Fig. 1G-I)".
(ii) Page 10, line 227: "Finally, constitutive CD38 KO mice showed similarly decreased synapse numbers in the mPFC (Appendix Fig. S3A-D) suggesting that CD38 in other types of cells does not contribute much to synaptogenesis". While I intuitively agree with the observation, the authors should test that this statement is true, using statistical tools to compare data from CD38 KO mice with data from CD38 AS-cKOP10 mice.
(iii) Page 13, line 282: "Treatment with SPARCL1 significantly recovered the decreased synapses in neurons cultured with CD38 KO-ACM up to 87.1% {plus minus} 0.09 of those with WT ACM (Fig 5A and B)." Here again, in my opinion, the statistics displayed on the histogram are not those needed to support the authors' conclusions. I believe that the authors should show the non-significant comparison between WT ACM with CD38 KO ACM + SPARCL1 to show they have a rescue of synapse number to WT values. On the other hand, if the test shows significant difference between the two groups, this would support the idea that SPARCL1 is an important synaptogenic factor, but is not sufficient for a complete rescue, indicating another molecule might well be acting in synergy with SPARCL1 during synaptogenesis (which is likely the case in reality). The outcome of this analysis should be commented on in the 'Discussion'.

General comments on the main text:
In general, I believe the phrasing and flow of ideas in the manuscript could still be improved (see previous comment on the 'Discussion'). This is because there are several instances where poor phrasing adversely impacts the clarity of an otherwise interesting study. I listed a few examples below, but this is not an exhaustive list and the authors should check throughout the manuscript for such issues:
(i) Page 2, line 40-41: Incorrect use of "on the other hand".
(ii) Page 2, line 47: The authors tend to make very strong, definitive statements, which in my opinion would benefit from being toned down. Is there anything wrong with using "We found...", "we demonstrate" (or equivalent) rather than asserting "Astrocytes show..."?
(iii) Page 3, line 63: Several repetitions of "and". It may read better to use commas instead.
(iv) Page 3, line 67: Is "Genomic-driven model of ASD" correct terminology? Would "transgenic model of ASD" be more appropriate?
(v) Page 3, lines 68 to 74: please split this long sentence into, at least, two shorter sentences.
(vi) Page 7, line 136: "these" not "this".
(vii) Page 7, line 139: Please consider starting the sentence with something other than "Whereas", which I do not think is grammatically correct.
(viii) P.8, line 168: "These results indicate that CD38 AS-cKOP10 mice exhibit deficits only in social memory". I would be more explicit and clearly explain that this result shows that CD38 AS-cKOP10 mice have no deficit in sociality, but a clear deficit in social memory. This would be clearer for readers not used to interpreting behavioral data.
(ix) Figure 6A: Please define the various cellular compartments.
(x) Page 16, line 388: "We also found 78.9% and 74.3% of reduced synapse number in the hippocampal CA1 and CA2 of the cKO, respectively (Fig EV5)." This sentence is confusing, On first reading, it appeared to me that the authors were arguing for a 78.9% reduction in synapse numbers in CA1 with CD38 cKO, when the reduction is in fact 20.1%. Maybe write "We found numbers of synapses in the hippocampal CA1 to be reduced to 78.9% of WT levels" (or something equivalent).
(xi) Page 18, line 421: "In contrast, the level of TSP1, another astroglial synaptogenic factor, was not changed in CD38 KO ACM by proteome and western blot analysis (Fig 4A and B)". In my opinion, this is another example of textual problems: words such as "as demonstrated by" are missing between "ACM" and "proteome".
(xii) Page 22 line 519: A word "day" is superfluous

We are very grateful to all the reviewers for their insightful comments and useful suggestions that have helped us improve our manuscript. Our point by point reply to the comments of the reviewers are below.

Referee #1

This reviewer believes the authors adequately addressed most of the previous comments and is excited by the overall findings of the work presented. However, two major concerns were not addressed in work shown. This reviewer does not feel the current work is ready for publication without addressing these two concerns.

Major Concern 1:

The first major concern is that the authors have not conducted a rescue experiment in their social memory task in their P10 CD38 cKO mice. The authors provide sufficient data to support the claim that CD38 cKO impairs Hevin secretion in astrocytes and that Hevin is sufficient to rescue synaptic phenotypes found in the CD38 cKO in vivo. However, the authors did not test the sufficiency of Hevin administered in early life to rescue the social memory deficit seen in the P10 CD38 cKO or global CD38 KO. This is an essential experiment to show that Hevin is the critical molecule secreted by astrocytes during this developmental period that regulates the establishment of the social memory circuit the authors are investigating.

This reviewer believes that because the mass spectrometry data has limited coverage of the secreted astrocyte proteome, proteins involved in the researcher's behavioral task may still be missed. For this reason, this reviewer recommends that the researchers administer Hevin either by overexpression or direct injection of the purified protein, just as they did in their synapse rescue experiment into control and CD38 cKO mice. By testing this cohort later using their social memory task, the authors could then show that despite the limited coverage of their mass spectrometry data, they still identified an astrocyte-derived factor sufficient to allow for proper social memory circuit function.

We thank the reviewer for this critical comment. As the reviewer commented, we believe that it is important to examine the role of asotrocytic SPARCL1 for the establishment of social memory in our model. Administration of recombinant SPARCL1 protein or developing a new mouse line by crossing astrocyte-specific SPARCL1 transgenic mice with CD38 AS-cKO^{P10} mice, together with behavioral test in adult, will

be necessary to complete these experiments. However, considering the possible involvement of synaptic impairment in broader social memory-related regions than those we examined for the rescue experiments in this study, we speculate that the strategy to use SPARCL1 protein may not be adequate, and we may need to develop a new mouse line above, which takes at least, another year to complete. Therefore, we would like to clarify this issue in future studies. We addressed this point as limitation of in this study in the Discussion section (page 18, line 441-444).

Major Concern 2:

The second major concern is that the authors limited their investigation to astrocyte-secreted factors. This reviewer believes that the authors provided sufficient data to support the claim that astrocyte secretion of Hevin is impaired in their CD38 cKO. However, the data presented also suggests that astrocytes are broadly dysregulated in their CD38 cKO, given that they find decreased S100b and GFAP with no changes in Sox9 or NDRG2 cell numbers. A previous concern from this reviewer was that astrocyte morphology was not tested in the CD38 cKO model.

If the authors performed the behavioral rescue experiment using Hevin in the CD38 cKO mice and found this was sufficient to recover normal social memory, then this reviewer feels that further investigation into astrocyte morphology would be interesting but not essential. However, without the rescue data, there is no way to determine if the synaptic phenotypes observed and the behavioral phenotypes observed are linked and dependent on the astrocyte secretion of Hevin. Previously published work has shown that astrocyte morphology depends on interaction with neurons and that impaired astrocyte morphology impairs synapse development. Therefore, investigating astrocyte morphology in the CD38 cKO model would be informative, especially if Hevin cannot rescue social memory.

This experiment is also within the technical capabilities of the lab, given that it could be accomplished by crossing their Glast-creERT2/CD38 floxed mice with a cre-dependent reporter line (such as Ai14) and providing a low dose of tamoxifen. Alternatively, the investigators could use previously published methods to sparsely transduce astrocytes with a cre virus + fluorescent protein under the GFAP promoter or through electroporation using a GFAP cre-plasmid + fluorescent protein.

We thank the reviewer for this critical comment. As previous studies have shown,

astrocyte morphology is closely linked with synapse formation (Jeff A Stogsdill et al., 2017). It is great interest to examine whether CD38 regulate synapse formation through modulating astrocyte morphology, as our previous study using immunohistochemistry of GFAP suggested altered shape of astroyctes in constitutive CD38 KO mice (Hattori et al., 2017). However, developing a new mouse line by crossing Ail4 mice with CD38 AS-cKO^{P10} mice followed by injection of Cre virus also may take another year to complete. Therefore, we would like to clarify this issue in future studies, and, in this study, focus on the role of astrocytic CD38 for synaptogenesis through astrocyte-derived factors.

Minor concerns:

Other minor problems with the work presented are:

1. While the investigators conducted RNA sequencing on cultured astrocytes from control and CD38 KO mice, this reviewer feels that the investigators might be confounding their effects because of the presence of serum in their astrocyte cultures. A cleaner experiment would be to cross their cKO line to a Cre-reporter line and FAC sort astrocytes for RNA sequencing.

As the reviewer commented, to obtain astrocytic gene profile in the brain, FACS-based cell isolation from CD38 AS-cKO^{P10} mice with astrocytic reporter gene might be better. Previous study has shown that 547 genes out of total 31000 genes were significantly different between acutely isolated-astrocytes from P1 brain and cultured astrocytes, respectively (Foo et al., 2012, Neuron). Although a part of genes may be affected by serum in culture medium, we think that our RNA-seq analysis is still informative to evaluate effect of CD38-deletion on gene expression in astrocytes. And we do not have the animal line which crossed with CD38 AS-cKO and Ai14 reporter mice at present. Therefore, in this study, we decide to use the present RNA-seq analysis to demonstrate the effect of astrocytic CD38 on calcium signaling (Figure 7).

2. The synapse images would benefit from changing to green and magenta (instead of red and green) to accommodate color-blind readers.

We changed colors from red and green to magenta and green in whole color figures.

3. The representative image for Figure 3 cKO PSD95 puncta does not show a reduction in PSD95 puncta. Perhaps a better representative image could be selected instead.

We changed the PSD95 puncta image of CD38 AS-cKO mice to a representative picture in Figure 3A.

Referee #2:

The authors have adequately addressed all my concerns. The manuscript is an important contribution to the field. I recommend publication of this manuscript.

Referee #3:

Astroglial CD38 regulates social memory and synapse formation in the developing brain
Hattori et al.
In this manuscript, Hattori et al. put forward evidence that CD38 knock out specifically in astrocytes leads to a deficit in social memory in adult mice, while other types of memory remain unaffected. This effect is present only if CD38 is knocked out around post-natal day 10 (CD38 AS-cKO P10), since KO induction at later age has no (overt) effects. Using Golgi stainings and immunohistochemistry, the authors found a reduced number of mature excitatory synapses in the medial prefrontal cortex (mPFC) and hippocampus of CD38 AS-cKO P10 mice. Switching to astrocyte-neuron co-culture models (in which the cells remain physically separated), the authors obtain evidence that the presence of CD38 is essential for secretion of a pro-synaptogenic factor. Using proteomics on astrocyte conditioned media (ACM) from CD38 KO and wild type (WT) primary astrocytes, they identify a putative factor, SPARCL1 (Hevin), which has been previously implicated in glutamatergic synaptogenesis. Crucially, SPARCL1 expression is downregulated in both isolated CD 38 KO astrocytes and the intact mPFC of CD38 AS-cKO P10. Interestingly, exogenous SPARCL1 can partially rescue the loss of synapses observed in both the in vitro and in vivo models used.

Applying pharmacological tools to their in vitro model, the authors further indicate that the CD38 signaling pathway, linked to downstream cADPR formation and calcium release from intracellular stores, is essential for SPARCL1 secretion from astrocytes. Finally, the authors analyzed the transcriptome of isolated CD38 KO astrocytes using bulk mRNA sequencing, and found that genes associated with calcium signaling pathways were downregulated in these cells, while inflammatory pathways genes were upregulated.

This is a revised manuscript of a version originally submitted via the Review Commons system. The authors have answered my main concerns, notably by demonstrating the astrocyte specificity of their transgenic mouse model, and providing more data on how the normalization of their western blot experiments was performed. The extension of some their conclusions to the hippocampus is interesting and increases the significance of the manuscript. While SPARCL1 has previously been implicated in synaptogenesis, to the best of my knowledge, this is the first time SPARCL1 and the CD38 signaling pathway have been linked, with astrocyte-specific CD38 ablation resulting in a specific functional deficit. While the experimental data has improved considerably, the largely rewritten 'Discussion' has some substantial issues which need to be responded to. If these are suitably addressed, alongside the other considerations listed below, then I would support publication in EMBO J, as the manuscript would be of interest both to the neuroscience and cell biology communities.

Major Comments:
(i) The data shown in Figure 4D, E and F is unconvincing. The blot data (Figure 4D) seems to show marked reduction in both SPARCL1 and CD38 expression at P70. However, this is not apparent in the quantification. I realize the data (Figure 4E, F) contains some low values, but perhaps a more representative blot would work better at this point. Likewise, the immunohistochemistry panels (Figure 4G and H) are also not particularly convincing.

We thank the reviewer for this suggestion. We changed the blot images in Figure 4E and F to representative images. The immunohistochemistry images of CD38 AS-cKO mice in Figure 4G was also replaced with representative ones. Furthermore, SPARCL1 positive or negative astrocytes were indicated by white or yellow arrowheads in Figure 4G. Figure legend of Figure 4G is also revised (page 48, line 1209-1210).

(ii) In general, I felt the 'Discussion' was poorly written. In my opinion, it contained several factual inaccuracies, as 2APB is an IP_3 antagonist, while Mondal and colleagues demonstrated a primary role for fibromodulin in filamentous actin stress fibre formation and not extracellular matrix assembly. It is also unclear to me how the authors can rule out a role for TSP1 in glutamatergic synapse assembly based on the data they present (which runs contrary to the large body of published studies). Comparison of SPARCL1 levels to other studies (Risher et al., 2014) only holds if exactly the same protocols are used. Given the small number of proteins detected in the ACM analysis, I would have thought a discussion on the limitation of their proteomic analysis would have been valid. Furthermore, I was disappointed at the lack of any attempt to link/speculate on how the comparatively small decrease in SPARCL1 secretion and synapse number could relate to a specific behavior. Finally, I believe that the reference to 'mitochondrial transfer to neurons in ischemic brains' was outside the scope of this study and, therefore, superfluous. Overall, it is my opinion that the 'Discussion' would benefit from some critical editing, preferably to include proof-reading by a native English speaker.

We apologize for inappropriate descriptions in the Discussion section. We described that blocking of IP_3 as well as cADPR/calcium signaling suppressed SPARCL1 release from astrocytes, which suggests involvement of calcium signaling for the SPARCL1 release (page 19, line 445-448). Regarding fibromodulin, although we described one of downregulated molecules by deletion of CD38 as potential regulator of synaptogenesis in the previous manuscript, it is quite unclear whether these molecules are related with synaptogenesis at present. Therefore, we deleted this part in the Discussion section.

As the reviewer commented, we cannot rule out involvement of the other molecules than SPARCL1 for the following reasons. 1) treatment with recombinant SPARCL1 did not fully recover the impaired synapse formation by the deletion of astroglial CD38 (Figure 5), 2) the number of identified proteins by our proteomics was fewer than those in other similar studies, 3) effect of astroglial CD38 on secretion of synaptogenic factors in the brain is still unclear. We described these limitations in our study and involvement of the other molecules in CD38-mediated synaptogenesis in the Discussion section (page 18, line 430-444).

We described two possible reason for social memory specific impairment in CD38 AS-cKO^{P10} mice in the Discussion section. 1) The slight synaptic reduction in the mPFC

is insufficient to induce mPFC-related behavior impairment. However, the synaptic reduction occurred in several critical regions for social memory such as mPFC, hippocampal CA1, and CA2. 2) Critical period of specific neural circuit formation is reported to be different in the postnatal mPFC (Klune et al, 2021). Although the critical period of social memory has not been clarified, spatiotemporal expression of astroglial CD38 may overlap with critical region and timing of neural circuit formation of social memory. We described this possible link between the slight synaptic reduction and specific impairment of social memory in the Discussion section (page 16, line 379-398)

As the reviewer suggested, we deleted the description regarding mitochondria transfer from astrocytes to neurons.

Proof-reading of the Discussion section was performed by a native English speaker.

(iii) Finally, based on all the data, can the authors justify the manuscript title 'Astroglial CD38 regulates social memory and synapse formation in the developing brain'. Wasn't social memory tested in adult mice?

We thank the reviewer for this suggestion. We changed the title as follows “The postnatal astrocytic CD38 regulates synapse formation and adult social memory”.

Minor comments:

In general, the layout of the text and figures has clearly improved. Nonetheless, small issues remain, such as axis labels being at variable distances from the axis (e.g. Fig. 6C vs. 6D), or the value "-1" in Fig. 7A being above the scale when other values are below. These small issues must be corrected throughout the manuscript before publication.

We thank the Reviewer for pointing out these issues. We checked the whole manuscript and revised all the issues.

Specific comments on science:

(i) Proof of specific CD38 KO in astrocytes: Page 6, line 132 and Figure EV2: If I understand correctly, Western Blots were performed with whole brain lysates, which is presumably why a residual level of CD38 is detected. In this instance, Western Blots performed using ACSA2-MACS purified astrocytes (as in Figure EV1), would be much more convincing for the story, particularly if they demonstrated complete

protein loss at 11 days post-tamoxifen injection, as I suspect they will do.

As the reviewer commented, western blot of the MACS purified astrocytes would clarify complete loss of astrocytic CD38 protein in the mPFC at P21. However, it is difficult to obtain enough amount of protein from these MACS-purified astrocytes to perform western blot. In Figure EV2, we performed western blots with mPFC brain samples. CD38 AS-cKO^{P10} mice showed reduction in CD38 protein level to 28.9% of those of control mice in the mPFC at P21 (Fig EV2B). On the other hand, in control mice *Cd38* mRNA expression level in ACSA2-MACS isolated astrocytes was 311% of that in ACSA negative cells (Fig EV1A). This results suggest that 24.3% of total *Cd38* expression is expressed in ACSA2 negative cells (other types of cells than astrocytes) in the control mice. Therefore, we estimate that the remaining 28.9% of CD38 protein in CD38 AS-cKO^{P10} mice is almost derived from non-astrocyte cells and most of the CD38 protein in astrocytes is already disappeared at P21.

(ii) Figure EV2A: It is unclear to this reviewer, why we are shown what seems to be three replicates per genotype in the blot image, while the quantification was done on four animals. Are these technical replicates? If yes, could the author state this in the figure legend? Also in the legend, the word 'analyses' in (A) should be 'analysis'.

We apologize for this confusing figure presentation. We changed these images to new images showing samples of two animals per genotype in Figure EV2A. And, we described “2 animals per genotype are shown” in the Figure legend of Figure EV2 (page 52, line 1273).

(iii) Figure 3 (and other relevant figures): Why was VGLUT1 used as the sole pre-synaptic marker? Are VGLUT2 levels so low in mPFC and hippocampus? A specific effect on VGLUT1 synapse assembly would be of great interest to the community. Why do there appear to be double bands in Figure 3E?

We thank the reviewer for this valuable comment. In the mPFC, thalamocortical axon terminals use VGlut2 (Hur et al., 2005, Kaneko et al., 2002). To examine effect of deletion of astrocytic CD38 on VGlut2 positive synapse formation in the mPFC, we performed immunohistochemistry with anti-VGlut2 and PSD95 antibodies. We found number of VGlut2 positive synapse in the mPFC of CD38 AS-cKO^{P10} was not changed. This result is described in the Results section (page 10, line 218-220) and shown in

Appendix Fig. S2.

In Figure 3E, we changed western blot images to show two samples per genotype. Figure legend of this figure also revised (page 47, line 1182).

(iv) Figure 4A source data: Why are the blots for TSP1 and beta-actin not included?

We apologize for insufficient data for this source data. We added these data in Figure 4A source data.

(v) Figure EV1: This is a most welcome addition. I would just indicate on the figure that the first row of bar plots comes from mPFC samples and the lower one from hippocampal samples.

We added these indications in Figure EV1.

Specific comments on statistics:
Personally, I have a small issue with the fact the authors are not clearly displaying, either in the manuscript or in a supplementary table, the complete results of their statistical tests. Yet, a number of conclusions are drawn from such 'non-significant' comparative tests. I think they should be reported, as the p-value of these results is important for the reader to interpret the data. A table might be the easiest way, to not overload the figures.

We thank the reviewer for this valuable suggestion. To show p-value of every comparison in this study, we made statistical summary of all figures as Dataset EV4.

Some examples:
(i) Figure 1: The authors display 'within group' statistical comparisons, which are, in general, interesting and needed, but wouldn't 'between group' comparisons also be of interest to reinforce the authors conclusions? This would allow the authors to legitimately write on p.8, line 173: "In these mice (CD38 AS-cKOP42), all social behaviors, sociability, social preference, and social memory were similar to those of ctrlP42 mice (Fig. 1G-I)".

We thank the reviewer for this valuable suggestion. We performed suggested statistical comparison between group. P-value of these comparison is also shown in Dataset EV4. We described the similar social behavior between ctrl^{P42} mice and CD38 AS-cKO^{P42} mice in the Results section (page 8, line 173).

(ii) Page 10, line 227: "Finally, constitutive CD38 KO mice showed similarly decreased synapse numbers in the mPFC (Appendix Fig. S3A-D) suggesting that CD38 in other types of cells does not contribute much to synaptogenesis". While I intuitively agree with the observation, the authors should test that this statement is true, using statistical tools to compare data from CD38 KO mice with data from CD38 AS-cKOP10 mice.

We added results of statistical test of comparison of synapse numbers in the mPFC between WT and ctrl^{P10} mice or CD38 KO and CD38 AS-cKO^{P10} mice in Dataset EV4 (Appendix S3D). Since we found no significant difference of this comparison, we keep the description above in the Results section (page 11, line 230-232).

(iii) Page 13, line 282: "Treatment with SPARCL1 significantly recovered the decreased synapses in neurons cultured with CD38 KO-ACM up to 87.1% {plus minus} 0.09 of those with WT ACM (Fig 5A and B)." Here again, in my opinion, the statistics displayed on the histogram are not those needed to support the authors' conclusions. I believe that the authors should show the non-significant comparison between WT ACM with CD38 KO ACM + SPARCL1 to show they have a rescue of synapse number to WT values. On the other hand, if the test shows significant difference between the two groups, this would support the idea that SPARCL1 is an important synaptogenic factor, but is not sufficient for a complete rescue, indicating another molecule might well be acting in synergy with SPARCL1 during synaptogenesis (which is likely the case in reality). The outcome of this analysis should be commented on in the 'Discussion'.

We thank the reviewer for this valuable comment. The comparison of synapse number between WT ACM and CD38 KO ACM + SPARCL1 showed no significant difference. We indicate this as N.S. in Figure 5B and p-value is shown in Dataset EV4. Furthermore, we also indicated no significant difference of mPFC synapse number between ctrl^{P10} and CD38 AS-cKO^{P10} + SPARCL1 in Figure 5D. Thus, SPARCL1 likely plays a major role in astroglial CD38-mediated synapse formation in the mPFC.

However, as we replied to major comment #2 of the Referee #3, we cannot rule out involvement of other molecules than SPARCL1 for the following reasons. 1) treatment with recombinant SPARCL1 did not fully recover the impaired synapse formation by the deletion of astroglial CD38 (Figure 5), 2) the number of identified proteins by our proteomics was fewer than those in other similar studies, 3) effect of astroglial CD38 on *in vivo* secretion of synaptogenic factors is still unclear. We described these limitations in this study and involvement of the other molecules in CD38-mediated synaptogenesis in the Discussion section (page 18, line 430-444).

General comments on the main text:

In general, I believe the phrasing and flow of ideas in the manuscript could still be improved (see previous comment on the 'Discussion'). This is because there are several instances where poor phrasing adversely impacts the clarity of an otherwise interesting study. I listed a few examples below, but this is not an exhaustive list and the authors should check throughout the manuscript for such issues:

We checked the whole manuscript, revised issues including the reviewer pointed out, and rewrite the Discussion section following the reviewer's comments. Proofreading was performed in the Discussion section. And, following issues the reviewer pointed out were also revised.

(i) Page 2, line 40-41: Incorrect use of "on the other hand".

(ii) Page 2, line 47: The authors tend to make very strong, definitive statements, which in my opinion would benefit from being toned down. Is there anything wrong with using "We found...", "we demonstrate" (or equivalent) rather than asserting "Astrocytes show..."?

(iii) Page 3, line 63: Several repetitions of "and". It may read better to use commas instead.

(iv) Page 3, line 67: Is "Genomic-driven model of ASD" correct terminology? Would "transgenic model of ASD" be more appropriate?

(v) Page 3, lines 68 to 74: please split this long sentence into, at least, two shorter sentences.

(vi) Page 7, line 136: "these" not "this".

(vii) Page 7, line 139: Please consider starting the sentence with something other than "Whereas", which I do not think is grammatically correct.

(viii) P.8, line 168: "These results indicate that CD38 AS-cKOP10 mice exhibit deficits only in social memory". I would be more explicit and clearly explain that this result shows that CD38 AS-cKOP10 mice have no deficit in sociality, but a clear deficit in social memory. This would be clearer for readers not used to interpreting behavioral data.

(ix) Figure 6A: Please define the various cellular compartments.

(x) Page 16, line 388: "We also found 78.9% and 74.3% of reduced synapse number in the hippocampal CA1 and CA2 of the cKO, respectively (Fig EV5)." This sentence is confusing, On first reading, it appeared to me that the authors were arguing for a 78.9% reduction in synapse numbers in CA1 with CD38 cKO, when the reduction is in fact 20.1%. Maybe write "We found numbers of synapses in the hippocampal CA1 to be reduced to 78.9% of WT levels" (or something equivalent).

(xi) Page 18, line 421: "In contrast, the level of TSP1, another astroglial synaptogenic factor, was not changed in CD38 KO ACM by proteome and western blot analysis (Fig 4A and B)". In my opinion, this is another example of textual problems: words such as "as demonstrated by" are missing between "ACM" and "proteome".

xii) Page 22 line 519: A word "day" is superfluous

2nd Revision - Editorial Decision **31st May 2023**

Dear Tsuyoshi,

Thank you for submitting your revised version. I have now had a chance to take a look at it and all looks good. I am therefore very pleased to accept the manuscript for publication here.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 χ2 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.

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Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Data availability section

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Matrials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

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Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)

If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	**Yes**	Materials and Methods
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	**Yes**	Materials and Methods
Include a statement about **blinding** even if no blinding was done.	**Yes**	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	Materials and Methods, Figure legends

Sample definition and in-laboratory replication	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Figure legends

Ethics

Ethics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	**Yes**	Matrials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	**Yes**	Data availability section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	