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Activation of the medial preoptic area (MPOA) ameliorates loss of maternal behavior in a Shank2 mouse model for autism

Stefanie Grabrucker, Jessica Pagano, Johanna Schweizer, Carolina Urrutia-Ruiz, Michael Schön, Kevin Thome, Günter Ehret, Andreas Grabrucker, Rong Zhang, Bastian Hengerer, Jürgen Bockmann, Chiara Verpelli, Carlo Sala, and Tobias Boeckers
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Corresponding author(s): Tobias Boeckers (tobias.boeckers@uni-ulm.de)

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1st Editorial Decision 22nd Jan 2020

Dear Tobias,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from their comments, the referees find the analysis interesting and suitable for publication in the EMBO Journal. They raise a number of different points that should be fairly straightforward to address and I would like to ask you to address them in a revised version. Regarding the points #2 and 3 raised by referee #3 => For point #2 please discuss this in the discussion - no need to generate a conditioned mutation of Shank2. Regarding point # 3, I agree with the point raised by the referee and would recommend a much more careful nuanced discussion regarding the links between Shank2 mutation, ASD and social behavior deficits.

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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

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- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
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Referee #1:

This manuscript by Grabrucker et al. suggests that the loss of social bonding (especially the affective bond between mother and pup) present in Shank2-deficient mice is due to the functional changes in the medial preoptic area (MPOA). The possibility that social bonding defect is due to hormonal, cognitive, or sensory deficits (i.e. olfactory) is ruled out via hormone concentration analysis, hormonal gene and receptor expression analysis, and a series of cognitive and sensory behavioral analysis. The authors then ameliorate the social bonding defect in Shank2 deficient mice via chemogenetic activation of MPOA neurons.

Given the scarcity of circuit information underlying autistic-like social deficits in mouse models of autism and the growing importance of mother-pup social bonding, this manuscript is providing an important step forward for our understanding of related circuit mechanisms. The experimental design and the quality of the data are quite solid.

Major comments:

1. The main message of this article that the suppressed activity of MPOA during social pup

exposure is most strongly supported by the social bonding 'restoration' with DREADD system (Figure 6). However, the n number is rather rather small to make a strong claim. The authors should perform power analysis to find out the optimal n number and increase the n number if necessary. Perhaps, this may even change the currently 'not significant' increases in pup retrieval, crouching, and nest building of KO normal saline vs. KO CNO to statistically significant, which will make the main conclusions of the article stronger. In the current manuscript, the most important factor of pup retrieval test (the number of pups retrieved) is not 'restored' but only partially ameliorated.

2. By activating MPOA neurons, the authors could improve social bonding in Shank2-/- mice. Authors mention in Discussion that a significant portion of c-fos positive neurons contains galanin and that galanin-positive neurons constitutes a major GABAergic neuronal population in MPOA. If reduced activity of GAL positive neurons would be the main cause of social bond impairment in Shank2-/- mice, activating GABAergic neurons in MPOA could also rescue abnormal behavior in Shank2-/- mice. I recommend the authors to chemogenetically activate GABAergic neurons in the MPOA to see if it also improves social bonding in Shank2-/- mice.

Minor comments:

1. The phrase 'causal mechanism of social impairment in Shank2 knockout mouse models of ASD has never been established' may be too strong to say. There are some evidence that NMDAR malfunction may underlie social deficits in Shank2 knockout mouse.

2. On page 8 '(Fig. 4H)' and '(Fig. 4I)' should be '(Fig. 2H)' and '(Fig. 2I)', respectively.

3. The method of CNO treatment (mentioned in figure 6A) is rather unclear. How long after the CNO treatment are the naïve WT and Shank2 female mice exposed to the pups (ex. immediately/ how many minutes after)? On the last day (day 5), how long after pup exposure do the subject mice perform pup retrieval test? The restoration experiment is currently missing from the method section and should also be addressed in detail.

4. The restoration experiments are only performed in naïve WT and Shank2 female mice chronically (repeated treatment of CNO for 5 days). If CNO treatment is done once, acutely, can social bonding related behavior be partially ameliorated in postpartum females?

5. Figure 3L: 'Progesteron' to 'Progesterone'

6. Figure 5H and Supplementary figure 4H: M. interaction graph looks different from the rest of the graphs (grooming, crouching, and nest building).

7. Figure 6 C-G: I would like the authors to also mark whether there is a significant or non-significant difference between WT+veh vs. KO+CNO. If there is a non-significant difference, this means that the adult-pup social bonding is indeed rescued. If not, this means that the adult-pup social bonding is only ameliorated.

Referee #2:

In this study by Grabrucker et al., the authors examine how loss of the Shank2 gene results in deficits in social behavior directed towards pups. The authors show that Shank2 KO mothers fail to take care of their pups, that this is not mediating by hormones, and that the pups develop normally

if they are fostered. Additionally, chemogenetic modulation of the mPOA increases maternal behavior and rescues pup retrieval. Overall, this is an interesting and important study. There are many critical control experiments that were performed, and the effects are quite robust. Overall, I am very enthusiastic about the potential publication of the manuscript. I have a few moderate concerns for the authors to consider, that can mostly be addressed with changes to the text.

1) In the DREADD experiments presented in Figure 6 the authors say they are manipulating neuroplasticity with this approach. This is not directly tested. Instead they should rephrase this to state they are activating the neurons or enhancing Gq signaling in the mPOA cells.
2) Another concern with the DREADD experiments - the authors claim they are activating glutamatergic mPOA neurons. However, it seems like their viral approach is not cell type specific, and that all infected mPOA neurons would be activated. Since the mPOA contains many GABAergic neurons as well as glutamatergic neurons, these findings should be rephrased in the context of global activation of mPOA instead of glutamatergic cells specifically.

In Figure 6, mcherry expression is labeled as RFP in the images.

The x-axis labels in figure panels of 2A,C,E,F,H,I are missing.

Referee #3:

General comments
This study entitled "activation of the medial preoptic area ameliorates loss of social bonding in Shank2 autism model" focuses on parental behavior performance of Shank2 mutant mice. The pup retrieval deficit of Shank2 mutant postpartum and virgin mice has been briefly reported elsewhere (Won, 2012), and can be anticipated from the multiple neurobehavioral abnormalities of Shank2 mutant mice. General health problems (high mortality, low body weight), heightened anxiety and hyperactivity found in shank2 mutants are all known to inhibit performance of parental behavior (for example, Crawly 2007; Kuroda 2011). Normal performance in object recognition, Y-maze tests and Social recognition of Shank2 mutants were also reported by the same research group (Schmeisser, 2012) (Ey, 2018).
The new findings of this study are showing normal mammary gland physiology, normal expression of hormones relevant for female reproductive functions, rather modest Shank2 expression in the brain circuit required for parental behavior, and the restoration of parental behavior by chemogenetic activation of the MPOA neurons in Shank2 mutants. These are surely of interest for researchers studying parental behavior, but might not be so for wider readership of this journal.
The figures and methods were clearly presented.

Major concerns
1 The obscure term "social bond" should be avoided
The term "social bond" (or sometimes "social attachment") is frequently used in this manuscript from the title, abstract and the results. However, the authors used this term very vague way, mixing up all kinds of positive social behaviors, including parental care, infant attachment to parents, pair bond, and adult attachment. For example, in the first sentence of the Introduction, the authors cited only one reference (Johnson and Young, 2015), the review paper about pair bond only. This is very

confusing, because these distinct contents of "social bonding behaviors" are evolutionary and mechanistically very different from each other, dependent on distinct neural circuitries and molecules. It is also questionable that the mouse parental behavior, which is regarded as a part of reproductive behaviors, includes "BOND formation", the term used in human psychology.
Thus, the use of more specific term "parental behavior" instead of "social bond" or "social bond behavior" is required at least in the title, abstract and results, because this manuscript only deals with parental behavior.

2 Parental care deficits might be caused secondarily from other Shank2 phenotypes
As mentioned above, the multiple abnormalities of Shank2 mutants may cause parental care deficits indirectly. To really deny this possibility, more specifically conditioned mutation of Shank2 showing only parental care deficits without hyperactivity, health problems or heightened anxiety should be created. Otherwise, the authors should explicitly raise this possibility in the Discussion.

3 The link between Shank2 mutation, autism and social behavior deficits
ASD children are shown to have similarly-secure attachment to their parents compared with IQ-matched children (for example, Sigman 1986, 1989), and numerous high-functioning ASD adults become parents. Even though their social skills have some problems in real life, they are not devoid of motivation to form social bond with others.
Also, while Shank2 was originally linked with autism, there are multiple reports that Shank2 mutations are also associated with neuropsychiatric disorders in general.
Therefore, it is too much of simplification to claim as if shank2 mutation, ASD and social behavior deficits are all definitive connection, as seen in a lot of descriptions in this manuscript. Rather their relationships are more variable. The manuscript should be revised to fully appreciate these facts.

Minor issues
-It should be acknowledged that the c-Fos expression in the MPOA and other brain areas after pup exposure is the result of, but not the cause of performance of parental behavior. Parental behavior starts much earlier (in a few minutes) than c-Fos protein expression (peaking at 2 hours). Even wild type mice show low c-Fos expression in the MPOA if these mice do not care for the pups. Therefore the lack of c-fos expression in the MPOA of pup-exposed Shank2 mutants should be natural consequence of their lack of parental behavior, but nothing more than that. With this regard, the claim found in the last sentence of page 15 Discussion is not agreeable.

- Parenting deficits of Shank2 mutants can be restored by chemogenetic activation of the MPOA. This fact supports that the major defect caused directly by Shank2 deletion is not in the MPOA per se, but in other brain areas activates MPOA neurons. This interpretation matches better the authors' finding that Shank2 is rather scarcely expressed in the MPOA.

- A possible confusion (?) between "pheromone" and "volatile odorant" is found in the first paragraph, page 8. Of note, mouse parental behavior is shown to be dependent on the main olfactory function, but not on the accessary olfactory system conveying pheromone.

- The method used for blood collection should be specified in the Method section.

- It wasn't clear to the reviewer why the described chemogenetic activation is limited to excitatory MPOA neurons as claimed in page 13, because both of the virus constructs used the hSyn promotor.

Response to reviews on manuscript EMBO: "Activation of the medial preoptic area ameliorates loss of social bonding in Shank2-/- ASD mice" by Grabrucker et al.

We would like to sincerely thank the referees for the time and effort they devoted to reviewing this manuscript. We appreciate the enthusiastic evaluations, scientific interest in the topic, and constructive comments and hope that our revision could significantly improve the manuscript.

Please see below a point-by-point response to the individual major and minor concerns:

Referee #1:

This manuscript by Grabrucker et al. suggests that the loss of social bonding (especially the affective bond between mother and pup) present in Shank2-deficient mice is due to the functional changes in the medial preoptic area (MPOA). The possibility that social bonding defect is due to hormonal, cognitive, or sensory deficits (i.e. olfactory) is ruled out via hormone concentration analysis, hormonal gene and receptor expression analysis, and a series of cognitive and sensory behavioral analysis. The authors then ameliorate the social bonding defect in Shank2 deficient mice via chemogenetic activation of MPOA neurons.
Given the scarcity of circuit information underlying autistic-like social deficits in mouse models of autism and the growing importance of mother-pup social bonding, this manuscript is providing an important step forward for our understanding of related circuit mechanisms. The experimental design and the quality of the data are quite solid.

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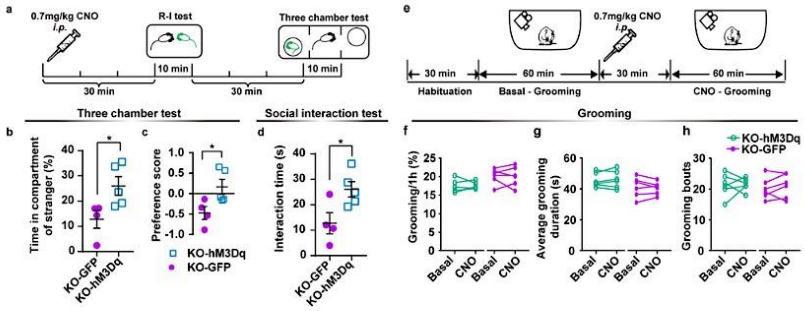
1.The main message of this article that the suppressed activity of MPOA during social pup exposure is most strongly supported by the social bonding 'restoration' with DREADD system (Figure 6). However, the n number is rather small to make a strong claim. The authors should perform power analysis to find out the optimal n number and increase the n number if necessary. Perhaps, this may even change the currently 'not significant' increases in pup retrieval, crouching, and nest building of KO normal saline vs. KO CNO to statistically significant, which will make the main conclusions of the article stronger. In the current manuscript, the most important factor of pup retrieval test (the number of pups retrieved) is not 'restored' but only partially ameliorated.

We based our sample size calculation on a previous publication with a similar experimental design (Fig. 18 d; Guo et al., 2019), where chemogenetic activation of

ACC pyramidal neurons via DREADDS-hm3Dq rescued social deficits in *Shank3*$^{-/-}$ deficient mice. In this publication, a significant effect on social interaction time has been reported after CNO treatment of *Shank3*$^{-/-}$ hm3Dq mice (two-tailed unpaired t-test, p= 0.032). We used the results obtained in this test for the power calculation because the key readouts of our study design are similar effect of CNO treatment on social behavior and effect of genotype (Shank deficient).



Supplementary Figure 18 Guo et al., 2019

Shank3$^{-/-}$*GFP*				*Shank3*$^{-/-}$*hm3Dq*			
mean	SEM	N	SD	mean	SEM	N	SD
12.81	4.27	4	8.54	26.01	3.11	5	6.95

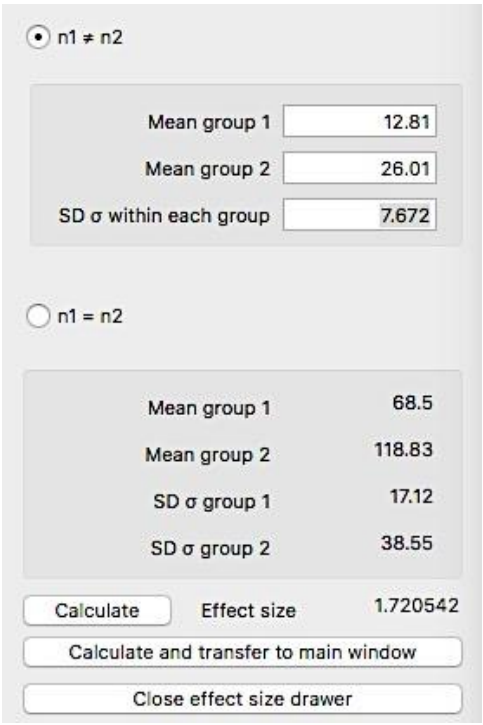
As shown in the table above, we do not have the same n between the treatment groups, so we calculated first the pooled standard deviation between the groups (using the converter at the following website: http://www.psychometrica.de/effect_size.html#transform).

13. Computation of the pooled standard deviation

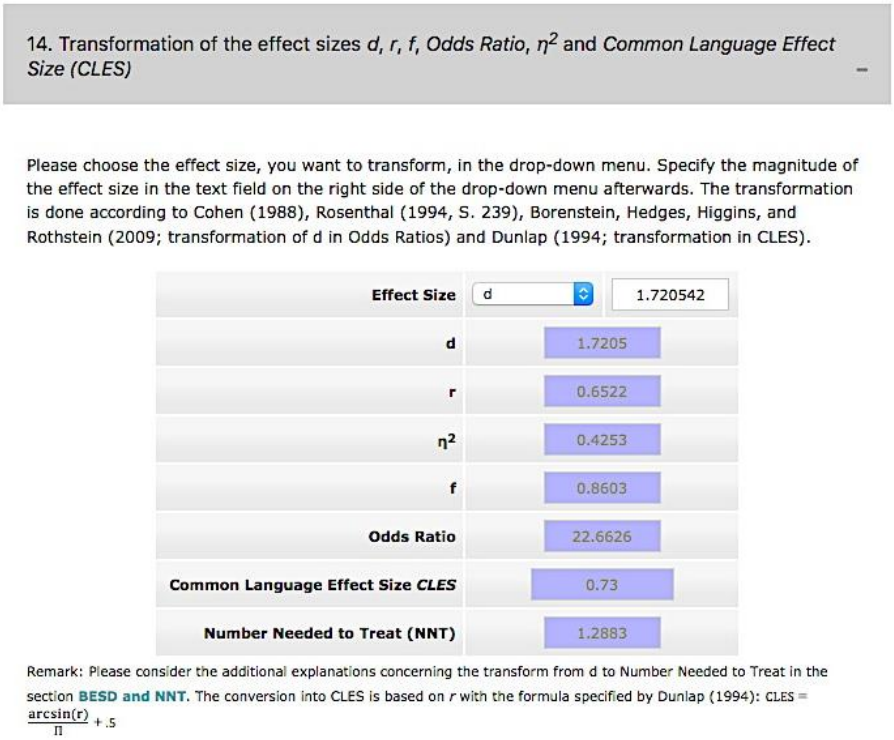
In order to compute Cohen's *d* and for other purposes, it is necessary to determine the mean (pooled) standard deviation. Here, you will find a small tool that does this for you. Different sample sizes are corrected as well and you can include up to 10 groups. Please specify the number before doing the calculation. If a value for the sample size is missing, the calculator only uses *sd* and does not correct for sample size.

Number of Groups ---	Standard Deviation (sd)	Sample Size (n)
Group 1	8.54	4
Group 2	6.95	5
Pooled Standard Deviation s_{pool}	7.672	

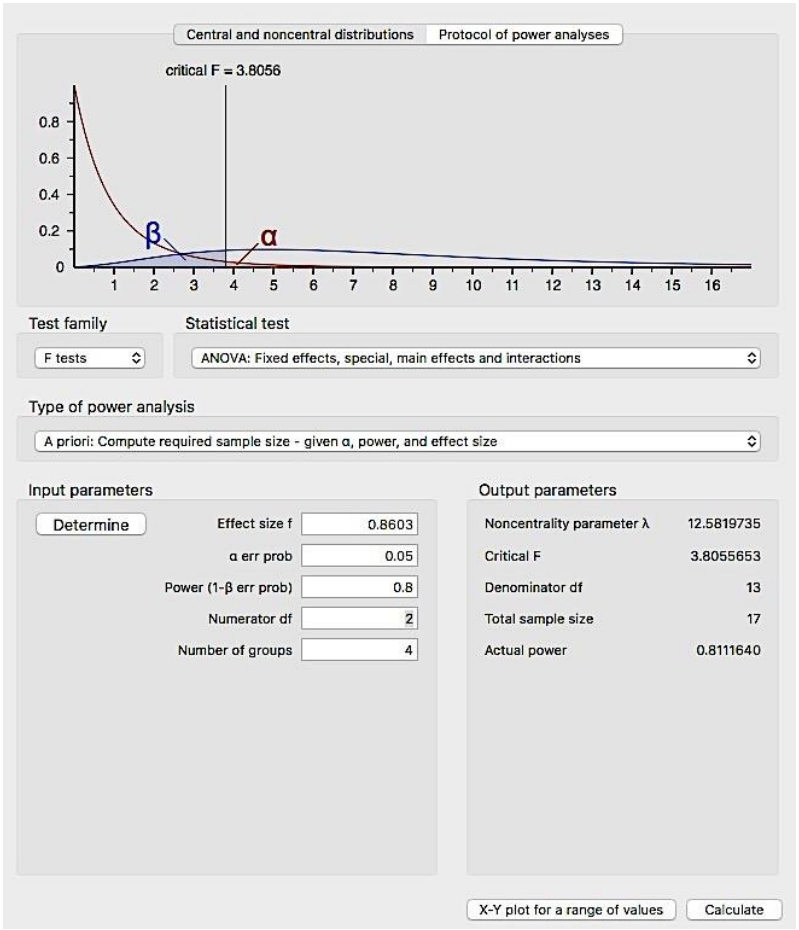
In the next step, we inserted the values of mean, pooled standard deviation in the G*power software to calculate the predicted **effect size (d)**. This calculation resulted in **d=1.720542**.



We converted the **d value** into **f value** (using the converter at the following website: http://www.psychometrica.de/effect_size.html#transform) and obtained **f=0.8603**



Our behavioral test has A x B factorial design with i=2 levels of A: treatment (Saline vs. CNO) and j=2 levels of B: genotype (WT vs. KO). Therefore, this design has in total four groups. A general assumption is that the dependent variable is normally distributed with identical variance in each group, allowing statistical analysis to be performed using a 2-way ANOVA design. We calculated the sample size given **f=0.8603**, **α=0.05**, and **power=0.8** for a **two-way ANOVA** design using G*Power software:



This calculation yields a total sample size of 17, divided by 4 (since four groups)=4.25. Thus **n=4.25** mice per group are necessary.

Reference:

Guo B, Chen J, Chen Q, Ren K, Feng D, Mao H, Yao H, Yang J, Liu H, Liu Y, Jia F, Qi C, Lynn-Jones T, Hu H, Fu Z, Feng G, Wang W, Wu S. Anterior cingulate cortex dysfunction underlies social deficits in Shank3 mutant mice. Nat Neurosci 2019; 22 (8): 1223-1234.

We based the n-number of our previous behavioral analysis on this power calculation because the key readout of our study design was "to determine the effect of chemogenetic activation via DREADDS-hm3Dq on social behavior in a Shank deficient mouse model".

Nevertheless, we agree with the reviewer, and to improve the quality of the manuscript, we now included more animals in our study. Unfortunately, due to COVID-19 regulations, the maintenance and generation of heterozygote breeding pairs in our animal facility were limited. However, we could add a new cohort of 2 animals per group to the existing dataset. Importantly, the addition of new animals per group increased the significance value for pup grooming and the number of

significant interactions for the different aspects of maternal behavior, supporting our hypothesis that the treatment had a specific effect on the genotype.

2. By activating MPOA neurons, the authors could improve social bonding in Shank2-/- mice. Authors mention in Discussion that a significant portion of c-fos positive neurons contains galanin and that galanin-positive neurons constitutes a major GABAergic neuronal population in MPOA. If reduced activity of GAL positive neurons would be the main cause of social bond impairment in Shank2-/- mice, activating GABAergic neurons in MPOA could also rescue abnormal behavior in Shank2-/- mice. I recommend the authors to chemogenetically activate GABAergic neurons in the MPOA to see if it also improves social bonding in Shank2-/- mice.

We appreciate this thoughtful suggestion. In our study we have not used a cell specific viral approach: We have activated GABAergic as well as glutamatergic neurons within the MPOA. This was not clearly described in the previous version of the manuscript. Therefore, we have we rephrased the result section of the manuscript to clarify that a global activation of MPOA rather than excitatory MPOA neurons only was performed. The idea of activating GABAergic neurons specifically is appealing and in line with recent literature of the circuit that shows a contribution of inhibitory neurons (Fang et al., 2018; Kohl et al., 2018). However, it would require us to generate new viral constructs and a new cohort of mice, which, unfortunately, is out of the scope in the present situation. To acknowledge the suggestion of the reviewer we added a sentence to the discussion that activating specifically GABAergic neurons would be an interesting experiment in future.

References:

Fang YY, Yamaguchi T, Song SC, Tritsch NX, Lin D. A Hypothalamic Midbrain Pathway Essential for Driving Maternal Behaviors. Neuron 2018; 98 (1): 192-207.e10.

Kohl J, Babayan BM, Rubinstein ND, Autry AE, Marin-Rodriguez B, Kapoor V, Miyamishi K, Zweifel LS, Luo L, Uchida N, Dulac C. Functional circuit architecture underlying parental behaviour. Nature 2018; 556 (7701): 326-331.

Minor comments:

1. The phrase 'causal mechanism of social impairment in Shank2 knockout mouse models of ASD has never been established' may be too strong to say. There are some evidence that NMDAR malfunction may underlie social deficits in Shank2 knockout mouse.

We agree with the reviewer. We, therefore, deleted the sentence in the Introduction of the manuscript and replaced it with: “Although the underlying mechanisms are still mostly unknown, it has been suggested that impaired NMDA receptor function might contribute to the development of social interaction deficits in *Shank2*$^{-/-}$ mice (Won et al., 2012)”.

2. On page 8 '(Fig. 4H)' and '(Fig. 4I)' should be '(Fig. 2H)' and '(Fig. 2I)', respectively.

We apologize for the error in the image sequence. We corrected the sequence on page 8 from Fig. 4H,I to Fig. 2H,I in the new version of the manuscript.

3. The method of CNO treatment (mentioned in figure 6A) is rather unclear. How long after the CNO treatment are the naïve WT and Shank2 female mice exposed to the pups (ex. immediately/ how many minutes after)?

We apologize for the lack of clarity, we exposed the mice to the pups 30 min after injection of vehicle or CNO. We added this information now to the figure legend (6A).

On the last day (day 5), how long after pup exposure do the subject mice perform pup retrieval test?

Subject mice perform the pup retrieval test for 20 min on day 5. We exposed *Shank2*$^{+/+}$ and *Shank2*$^{-/-}$ mice on each day 1-5 for 20 min to the pups. Maternal behavior was analyzed in the final 20 min of pup exposure on day 5 (test day, 24 h after the last pup exposure, which means after 4 days continuous pup presentation). We have added this information to the manuscript's material and method section (Behavioral experiments after DREADD).

The restoration experiment is currently missing from the method section and should also be addressed in detail.

We added this information now in detail to the material and method section of the manuscript (Stereotaxic injections and DREADD behavioral experiments).

4. The restoration experiments are only performed in naïve WT and Shank2 female mice chronically (repeated treatment of CNO for 5 days). If CNO treatment is done once, acutely, can social bonding related behavior be partially ameliorated in postpartum females?

We thank the reviewer for his/her suggestion. Indeed, the chemogenetic activation of MPOA in postpartum females would be a fascinating experiment for our study, and we consider investigating this in detail in future studies. Unfortunately, the experiment is time-consuming, and in the current situation challenging for us. It requires us to breed a new cohort of *Shank2*$^{+/+}$ and *Shank2*$^{-/-}$ mice, which need to reach adulthood, pregnancy, and go through stereotaxic injections. We estimate the proposed experiments to take up to 1 year, which is unfortunately out of the scope in the current situation.

5. Figure 3L: 'Progesteron' to 'Progesterone'

We thank the reviewer for his/her thorough revision of our manuscript. We have corrected ‘Progesteron’ to ‘Progesterone’ in Figure 3L.

6. Figure 5H and Supplementary figure 4H: M. interaction graph looks different from the rest of the graphs (grooming, crouching, and nest building).

This has been corrected. All graphs in Figure 5H and Supplementary Figure 4H (now Supplementary Figure 5H, (grooming, crouching, nest building, M. interaction)) have now the same color scheme and layout. We also updated this in Figure 3G, and Figure 6G to keep the colors assigned in the graphs uniform within the manuscript.

7. Figure 6 C-G: I would like the authors to also mark whether there is a significant or non-significant difference between WT+veh vs. KO+CNO. If there is a non-significant difference, this means that the adult-pup social bonding is indeed rescued. If not, this means that the adult-pup social bonding is only ameliorated.

We thank the reviewer for his/her suggestion and revision of our manuscript. We now marked a significant or non-significant difference between WT+veh vs. KO+CNO in Figure 6 C-G.

Referee #2:

In this study by Grabrucker et al., the authors examine how loss of the Shank2 gene results in deficits in social behavior directed towards pups. The authors show that Shank2 KO mothers fail to take care of their pups, that this is not mediating by hormones, and that the pups develop normally if they are fostered. Additionally, chemogenetic modulation of the mPOA increases maternal behavior and rescues pup retrieval. Overall, this is an interesting and important study. There are many critical control experiments that were performed, and the effects are quite robust. Overall, I am very enthusiastic about the potential publication of the manuscript. I have a few moderate concerns for the authors to consider, that can mostly be addressed with changes to the text.

1) In the DREADD experiments presented in Figure 6 the authors say they are manipulating neuroplasticity with this approach. This is not directly tested. Instead they should rephrase this to state they are activating the neurons or enhancing Gq signaling in the mPOA cells.

We agree with the reviewer. We rephrased the sentence, "allowing us to manipulate neuroplasticity in MPOA neurons selectively" to "allowing us to activate neurons in the MPOA"

2) Another concern with the DREADD experiments - the authors claim they are activating glutamatergic mPOA neurons. However, it seems like their viral approach is not cell type specific, and that all infected mPOA neurons would be activated. Since the mPOA contains many GABAergic neurons as well as glutamatergic neurons, these findings should be rephrased in the context of global activation of mPOA instead of glutamatergic cells specifically.

We agree with the suggestions of the reviewer. Therefore, we changed the following sentences in the results section of the manuscript:

- We deleted “excitatory” from the subheading “targeted activation of excitatory MPOA neurons in the hypothalamus reinforces social bonding behavior in Shank2 $^{-/-}$mice”.
- We deleted "selectively excitatory" from the sentence "we decided to target selectively excitatory MPOA neurons."
- We deleted “glutamatergic” from the sentence “chemogenetic activation of glutamatergic MPOA neurons alone”

Also, we edited the figure legend of Figure 6:

- We rephrased the subheading of the figure legend from “DREADD-based excitation of MPOA neurons ameliorates social bonding in Shank2 $^{-/-}$mice” to “DREADD-based activation of MPOA neurons ameliorates social bonding in Shank2 $^{-/-}$mice."

Furthermore, we

- deleted "glutamatergic" from the sentence "chemogenetic activation of MPOA neurons in the manuscript's Introduction section.

In Figure 6, mcherry expression is labeled as RFP in the images.

We apologize for this error. We replaced RFP with mcherry in Figure 6 as well as in the Figure legend.

The x-axis labels in figure panels of 2A,C,E,F,H,I are missing

We thank the reviewer for the thorough evaluation of our manuscript. We now added the x-axis labels to the figures as described below:

- Figure 2A,C (postnatal days)
- Figure 2E,F (# of odor presentation, we also included numeration)
- Figure H (identical object, novel object)
- Figure I (Y Maze)

Referee #3: General comments

This study entitled "activation of the medial preoptic area ameliorates loss of social bonding in Shank2 autism model" focuses on parental behavior performance of Shank2 mutant mice. The pup retrieval deficit of Shank2 mutant postpartum and virgin mice has been briefly reported elsewhere (Won, 2012), and can be anticipated from the multiple neurobehavioral abnormalities of Shank2 mutant mice. General health problems (high mortality, low body weight), heightened anxiety and hyperactivity found in shank2 mutants are all known to inhibit performance of parental behavior (for example, Crawly 2007; Kuroda 2011). Normal performance in object recognition, Y-maze tests and Social recognition of Shank2 mutants were also reported by the same research group (Schmeisser, 2012) (Ey, 2018). The new findings of this study are showing normal mammary gland physiology, normal expression of hormones relevant for female reproductive functions, rather modest Shank2 expression in the brain circuit required for parental behavior, and the restoration of parental behavior by chemogenetic activation of the MPOA neurons in Shank2 mutants. These are surely of interest for researchers studying parental behavior, but might not be so for wider readership of this journal. The figures and methods were clearly presented.

Major concerns

1 The obscure term "social bond" should be avoided The term "social bond" (or sometimes "social attachment") is frequently used in this manuscript from the title, abstract and the results. However, the authors used this term very vague way, mixing up all kinds of positive social behaviors, including parental care, infant attachment to parents, pair bond, and adult attachment. For example, in the first sentence of the Introduction, the authors cited only one reference (Johnson and Young, 2015), the review paper about pair bond only. This is very confusing, because these distinct contents of "social bonding behaviors" are evolutionary and mechanistically very different from each other, dependent on distinct neural circuitries and molecules. It is also questionable that the mouse parental behavior, which is regarded as a part of reproductive behaviors, includes "BOND formation", the term used in human psychology. Thus, the use of more specific term "parental behavior" instead of "social bond" or "social bond behavior" is required at least in the title, abstract and results, because this manuscript only deals with parental behavior.

We acknowledge that there was a lack of clarity in the manuscript. We agree that the reference from Johnson and Young, 2015, which deals with pair-bond formation only, should be complemented by further references focusing on the different forms of social bonding behavior (parent-infant, filial, pair).
However, there is a consensus in the literature that the mother-infant interaction is classified as social bonding and social attachment behavior in mice (Insel et al., 1997; Insel and Young, 2001; Broad et al., 2006; Mogi et al., 2011; Nagasawa et al., 2012; Roth et al., 2013). Recent studies provide direct evidence that mother-infant

bonding in mice exists (Mogi et al., 2017; Lassi and Tucci, 2017) and that it is even persistent beyond the nursing period (Stroobants et al., 2020). Additionally, similar as in humans, disruption of mother-infant interactions in mice and rats have been shown to affect adult offspring sociability and induce long-lasting changes in neuronal development (Branchi et al., 2006; Starr-Phillips et al., 2014). We believe that there is sufficient evidence that mother-infant interaction in mice can not just be regarded as a part of the reproductive act and can give valuable insight into mechanisms in humans how attachment is formed in ASD.

We agree with the reviewer that mice do not form complex social bonding with the same sophistication as humans. However, they become socially attached to their infants, and the proximate mechanisms underlying various forms of positive social behaviors, including pair bonding and maternal-infant bonding, rely on common neural and endocrine systems that are highly evolutionary conserved between mammals (Insel and Young, 2001; Carter et al., 2002; Broad et al., 2006; Numan and Young et al., 2016). Based on the current literature, we prefer not to restrict the terminology social bonding or social attachment to humans only.

References:

Branchi I, D'Andrea I, Fiore M, Di Fausto V, Aloe L, Alleva E. Early social enrichment shapes social behavior and nerve growth factor and brain-derived neurotrophic factor levels in the adult mouse brain. Biol Psychiatry 2006; 60 (7): 690-6.

Broad KD, Curley JP, Keverne EB. Mother-infant bonding and the evolution of mammalian social relationships. Philos Trans R Soc Lond B Biol Sci 2006; 361 (1476): 2199-214.

Carter SC, Keverne EB. The neurobiology of social affiliation and pair bonding. Brain and Behavior 2002; 1: 299-339.

Insel TR. A neurobiological basis of social attachment. Am J Psychiatry 1997; 154 (6): 726-35.

Insel TR, Young LJ. The neurobiology of attachment. Nat Rev Neurosci 2001; 2 (2): 129-36.

Lassi G, Tucci V. Gene-environment interaction influences attachment-like style in mice. Genes Brain Behav 2017; 16 (6): 612-618.

Mogi K, Takakuda A, Tsukamoto C, Ooyama R, Okabe S, Koshida N, Nagasawa M, Kikusui T. Mutual mother-infant recognition in mice: The role of pup ultrasonic vocalizations. Behav Brain Res 2017; 325 (Pt B): 138-146.

Mogi K, Nagasawa M, Kikusui T. Developmental consequences and biological significance of mother-infant bonding. Prog Neuropsychopharmacol Biol Psychiatry 2011; 35 (5): 1232-41.

Nagasawa M, Okabe S, Mogi K, Kikusui T. Oxytocin and mutual communication in mother-infant bonding. Front Hum Neurosci 2012; 6: 31.

Numan M, Young LJ. Neural mechanisms of mother-infant bonding and pair bonding: Similarities, differences, and broader implications. Horm Behav 2016; 77: 98-112.

Roth TL, Raineki C, Salstein L, Perry R, Sullivan-Wilson TA, Sloan A, Lalji B, Hammock E, Wilson DA, Levitt P, Okutani F, Kaba H, Sullivan RM. Neurobiology of secure infant attachment and attachment despite adversity: a mouse model. Genes Brain Behav 2013; 12 (7): 673-80.

Starr-Phillips EJ, Beery AK. Natural variation in maternal care shapes adult social behavior in rats. Dev Psychobiol 2014; 56 (5): 1017-26.

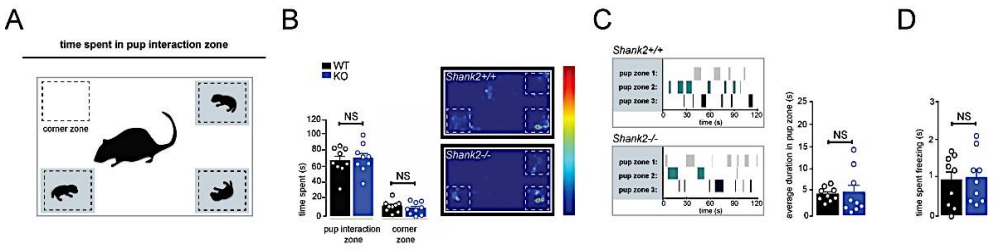
Stroobants S, Creemers J, Bosmans G, D'Hooge R. Post-weaning infant-to-mother bonding in nutritionally independent female mice. PLoS One 2020; 15 (1): e0227034.

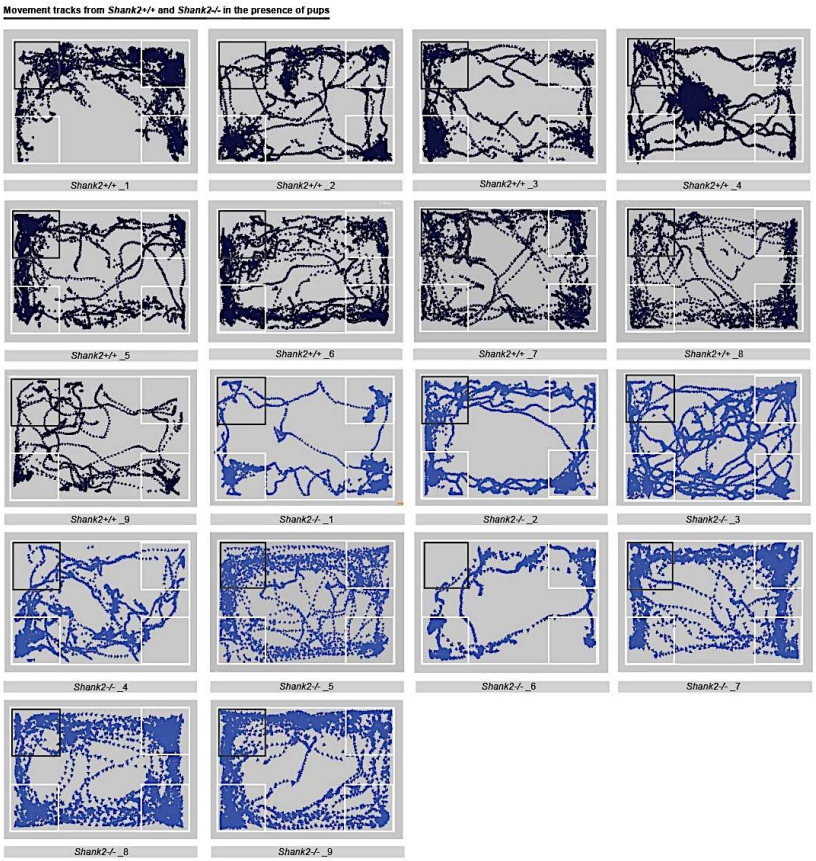
2 Parental care deficits might be caused secondarily from other Shank2 phenotypes As mentioned above, the multiple abnormalities of Shank2 mutants may cause parental care deficits indirectly. To really deny this possibility, more specifically conditioned mutation of Shank2 showing only parental care deficits without hyperactivity, health problems or heightened anxiety should be created. Otherwise, the authors should explicitly raise this possibility in the Discussion.

The reviewer's suggestion to investigate the effect of parenteral care deficits in a conditional *Shank2*$^{-/-}$ is appealing. Unfortunately, the generation and full behavioral characterization of a conditional *Shank2*$^{\Delta ex7-/-}$ mouse line are out of the present study's scope. We agree that constitutive *Shank2*$^{-/-}$ mice exhibit multiple ASD-

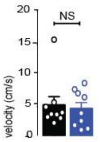
related comorbidities and that hyperactivity, health problems, and anxiety were not explicitly addressed in the prior version of the manuscript. Thus, as proposed by the reviewer, we flagged this possibility now in the discussion section. To clarify this further, we tried to determine the effect of anxiety, hyperactivity, and general health problems in the context of parenteral behavior in *Shank2*$^{+/+}$ and *Shank2*$^{-/-}$ mice with our current dataset.

- **Anxiety:** To evaluate if the pups induce social anxiety in *Shank2*$^{-/-}$ mice, we tracked naive *Shank2*$^{+/+}$ and *Shank2*$^{-/-}$ mice during the first two minutes of social exposure toward the pups (trial 1) using EthoVision XT software. We measured the time the mice spent in the "pup interaction zones" as well as "corner zone containing no pups" (Fig. A). We found that *Shank2*$^{-/-}$ mice did not avoid the pup interaction zones (indicated by the movement tracking path of each animal) and spent the same amount of time in the pup interactions zones as *Shank2*$^{+/+}$ mice (Fig. B). We also measured the average time that *Shank2*$^{-/-}$ mice spent in the "pup interacting zone" (Fig. C) as well as the time spent freezing (Fig. D) during this social investigation phase. We did not detect significant differences between both genotypes.

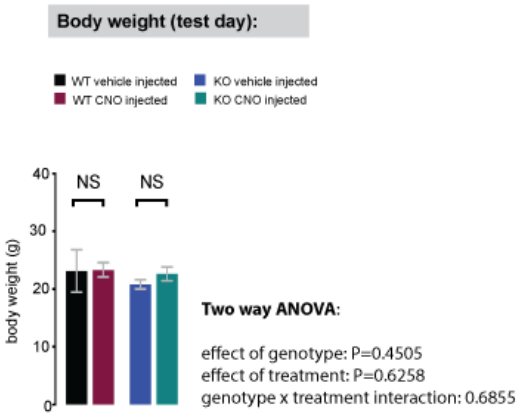




- **Hyperactivity**: We observed previously that *Shank2*$^{-/-}$ mice become extremely hyperactive when introduced into a new environment (e.g., in the open field test), whereas their activity levels declined in a familiar environment. We performed the pup retrieval test in their habituated homecage and exposed *Shank2*$^{-/-}$ mice for 5 days (trial 1-5) to the pups. We tracked the distance moved on the final day (test day, trial 5) using EthoVision XT software and calculated the average velocity (please see graph below) when *Shank2*$^{+/+}$ and *Shank2*$^{-/-}$ mice are not engaging in maternal behavior. We did not find significant differences between both genotypes. We also closely monitored the regular activity pattern of postpartum *Shank2*$^{-/-}$ mice in general in their home cage previously. We found that postpartum *Shank2*$^{-/-}$ mice also ignored the pups when they settled down on their nest location (see pictures below).



- **Bodyweight:** We agree that adult *Shank2*$^{-/-}$ mice are characterized by a significant reduction in body weight compared to *Shank2*$^{+/+}$ mice. However, they were capable of displaying parenteral behavior after CNO treatment, which might implicate that the general physiology of *Shank2*$^{-/-}$ mice does not limit the capability of *Shank2*$^{-/-}$ mice to engage in maternal behavior. We compared the bodyweight of vehicle-injected to the bodyweight of CNO treated *Shank2*$^{+/+}$ and *Shank2*$^{-/-}$ mice and found no significant change.



- **Mortality rate**: We agree with the reviewer that *Shank2*$^{-/-}$ mice exhibit a higher lethality rate during their very early development. However, we have not observed this effect in our laboratory in adult *Shank2*$^{-/-}$ mice.
  There is a consensus in the literature that the health & physiology of *Shank2*$^{-/-}$ mice is not in such a way impaired, which might limit their capability to participate in behavioral tests or engage in social behavior. This is evident by various previous publications conducting SHIRPA tests before the mouse model's initial behavioral characterization (Schmeisser et al., 2012) or performing a detailed social-interaction real-time analysis of *Shank2*$^{-/-}$ mice over several constitutive days (de Chaumont et al., 2019).

References:

de Chaumont F, Ey E, Torquet N, Lagache T, Dallongeville S, Imbert A, Legou T, Le Sourd AM, Faure P, Bourgeron T, Olivo-Marin JC. Real-time analysis of the behaviour of groups of mice via a depth-sensing camera and machine learning. Nat Biomed Eng 2019; 3 (11): 930-942.

Schmeisser MJ, Ey E, Wegener S, Bockmann J, Stempel AV, Kuebler A, Janssen AL, Udvardi PT, Shiban E, Spilker C, Balschun D, Skryabin BV, Dieck St, Smalla KH, Montag D, Leblond CS, Faure P, Torquet N, Le Sourd AM, Toro R, Grabrucker AM, Shoichet SA, Schmitz D, Kreutz MR, Bourgeron T, Gundelfinger ED, Boeckers TM. Autistic-like behaviours and hyperactivity in mice lacking ProSAP1/Shank2. Nature 2012; 486 (7402): 256-60.

3 The link between Shank2 mutation, autism and social behavior deficits ASD children are shown to have similarly-secure attachment to their parents compared with IQ-matched children (for example, Sigman 1986, 1989), and numerous high-functioning ASD adults become parents. Even though their social skills have some problems in real life, they are not devoid of motivation to form social bond with others.

We thank the reviewer for this constructive comment and for bringing up this important point. Studies provide evidence that children with ASD can establish secure attachment to their primary caregivers (Sigman and Mundy 1989). However, results are conflicting (Rutgers et al., 2004) and are heavily influenced by ASD phenotypes' heterogeneity and severity. It has been suggested that attachment in ASD may not be as prevalent as in typically developing children (Kahane and El-Tahir, 2015). Some studies have reported that children with ASD show higher rates of attachment insecurity or display disorganized attachment patterns compared to their control groups (Rutgers et al., 2004; Naber et al., 2007; Capps et al., 1994), and it has been suggested that the severity of ASD and mental retardation could be an important factor. However, other studies have shown that mental retardation did not interfere with the association between autistic symptoms in the social domain and attachment security (van Ijzendoorn MH, 2007). Further studies have demonstrated that especially high functioning ASD children display low attachment security (Rutgers et al., 2007) and that only a minority of adults with high functioning autism develop secure attachment behaviour, which was not related to IQ and theory of mind (Taylor et al., 2008). Besides, avoidant attachment patterns occur (Gallitto and Leth-Steensen, 2015; Lamport and Turner 2014).
In addition to ASDs being a heterogeneous group of disorders, attachment may manifest differently in ASD and can be challenging to evaluate. For example, typical attachment behaviors such as proximity seeking and contact maintenance may have different functions in ASD. Proximity seeking in children with ASD may mean proximity to the familiar (a familiar person or a familiar object) and avoidance of an uncertain, unfamiliar environment. Our study has also shown that the circuit is functionally not wholly compromised. *Shank2*$^{-/-}$ mice can display social attachment, however, only with increased activation of the MPOA. We assume that similarly, children with ASD may form social attachment to their primary caregivers with an increased input to the MPOA (during long-term extensive, sensory exposure). However, attachment formation in ASD might be impaired or delayed, which might have important implications for the refinement of neuronal pathways during critical windows of social brain development (Vivanti and Nuske, 2017).

We hope the present study will provide a basis for further research in this direction. To address the reviewer's considerations, the conclusion section of the Discussion underwent a major revision. We tried to integrate our results into the current literature about the role of attachment in ASD and high functioning autism.

References:

Capps L, Sigman M, Mundy P. Attachment security in children with autism. Development and Psychopathology 1994; 6: 249-261.

Gallitto E and Leth-Steensen C. Autistic traits and adult attachment styles: Personality and Individual Differences 1994; 79: 63–67.

Kahane L, El-Tahir M. Attachment behavior in children with Autistic Spectrum Disorders. Advances in Mental Health and Intellectual Disabilites. 2015; 9 (2) pp. 79-89.

Lamport D, Turner LA. Romantic attachment, empathy, and the broader autism phenotype among college students. J Genet Psychol 2014; 175 (3-4): 202-13.

Naber FB, Swinkels SH, Buitelaar JK, Bakermans-Kranenburg MJ, van IJzendoorn MH, Dietz C, van Daalen E, van Engeland H. Attachment in toddlers with autism and other developmental disorders. J Autism Dev Disord 2007; 37 (6): 1123-38.

Rutgers AH, Bakermans-Kranenburg MJ, van Ijzendoorn MH, van Berckelaer-Onnes IA. Autism and attachment: a meta-analytic review. J Child Psychol Psychiatry 2004; 45 (6): 1123-34.

Rutgers AH, van Ijzendoorn MH, Bakermans-Kranenburg MJ, Swinkels SH, van Daalen E, Dietz C, Naber FB, Buitelaar JK, van Engeland H. Autism, attachment and parenting: a comparison of children with autism spectrum disorder, mental retardation, language disorder, and non-clinical children. J Abnorm Child Psychol 2007; 35 (5): 859-70.

Sigman M, Mundy P. Social attachments in autistic children. J Am Acad Child Adolesc Psychiatry 1989; 28 (1): 74-81.

Taylor EL, Target M, Charman T. Attachment in adults with high-functioning autism. Attach Hum Dev 2008; 10 (2): 143-63.

van Ijzendoorn MH, Rutgers AH, Bakermans-Kranenburg MJ, Swinkels SH, van Daalen E, Dietz C, Naber FB, Buitelaar JK, van Engeland H. Parental sensitivity and attachment in children with autism spectrum disorder: comparison with children with mental retardation, with language delays, and with typical development. Child Dev 2007; 78 (2): 597-608.

Vivanti G, Nuske HJ. Autism, attachment, and social learning: Three challenges and a way forward. Behav Brain Res 2017; 325 (Pt B): 251-259.

Similarly, Also, while Shank2 was originally linked with autism, there are multiple reports that Shank2 mutations are also associated with neuropsychiatric disorders in general. Therefore, it is too much of simplification to claim as if shank2 mutation, ASD and social behavior deficits are all definitive connection, as seen in a lot of descriptions in this manuscript. Rather their relationships are more variable. The manuscript should be revised to fully appreciate these facts.

We acknowledge that the connection between Shank2 mutations, ASD, social behavior, and other neuropsychiatric disorders is complex and that this complexity was not clearly outlined in the previous version of the manuscript. Therefore, we extended the introduction of the manuscript and added references for the association of the human SHANK2 gene to schizophrenia, developmental delay, and intellectual disability. We hope this will better indicate the complexity of Shank2 mutations and their association with different neuropsychiatric disorders.

Although Shank2 mutations are associated with other neuropsychiatric disorders in general, we would like to mention that it is so far unclear how the various mutations in SHANK2 might predispose differently to ASD, intellectual disability, and schizophrenia (Homann et al., 2016). Different SHANK2 mutations might translate into "loss of functions" or "gain of functions," or truncated proteins that might regulate only particular aspects of the wildtype protein, resulting in a wide range of phenotypes.
The *Shank2*$^{\Delta ex7}$ KO mouse model used in the present study was generated by the deletion of exon 16, which resembles PDZ domain-microdeletions found specifically in human ASD patients (Berkel et al., 2010). In addition, it has been shown that

Shank2$^{\Delta ex7}$ KO mice recapitulate the full ASD phenotype (Schmeisser et al., 2012). We believe, therefore, that *Shank2*$^{\Delta ex7}$ KO mice model the "ASD aspect" of Shank2 mutations.

References:

Berkel S, Marshall CR, Weiss B, Howe J, Roeth R, Moog U, Endris V, Roberts W, Szatmari P, Pinto D, Bonin M, Riess A, Engels H, Sprengel R, Scherer SW, Rappold GA. Mutations in the SHANK2 synaptic scaffolding gene in autism spectrum disorder and mental retardation. Nat Genet 2010; 42 (6): 489-91.

Homann OR, Misura K, Lamas E, Sandrock RW, Nelson P, McDonough SI, DeLisi LE. Whole-genome sequencing in multiplex families with psychoses reveals mutations in the SHANK2 and SMARCA1 genes segregating with illness. Mol Psychiatry 2016; 21 (12): 1690-1695.

Schmeisser MJ, Ey E, Wegener S, Bockmann J, Stempel AV, Kuebler A, Janssen AL, Udvardi PT, Shiban E, Spilker C, Balschun D, Skryabin BV, Dieck St, Smalla KH, Montag D, Leblond CS, Faure P, Torquet N, Le Sourd AM, Toro R, Grabrucker AM, Shoichet SA, Schmitz D, Kreutz MR, Bourgeron T, Gundelfinger ED, Boeckers TM. Autistic-like behaviours and hyperactivity in mice lacking ProSAP1/Shank2. Nature 2012; 486 (7402): 256-60.

Minor issues

It should be acknowledged that the c-Fos expression in the MPOA and other brain areas after pup exposure is the result of, but not the cause of performance of parental behavior. Parental behavior starts much earlier (in a few minutes) than c-Fos protein expression (peaking at 2 hours). Even wild type mice show low c-Fos expression in the MPOA if these mice do not care for the pups. Therefore the lack of c-fos expression in the MPOA of pup-exposed Shank2 mutants should be natural consequence of their lack of parental behavior, but nothing more than that. With this regard, the claim found in the last sentence of page 15 Discussion is not agreeable.

We agree with the reviewer. Therefore we deleted the sentence "since essential circuit-specific activation patterns are blocked at a defined site, namely the medial preoptic area (MPOA)" from the discussion section.

Parenting deficits of Shank2 mutants can be restored by chemogenetic activation of the MPOA. This fact supports that the major defect caused directly by Shank2 deletion is not in the MPOA per se, but in other brain areas activates MPOA neurons. This interpretation matches better the authors' finding that Shank2 is rather scarcely expressed in the MPOA.

We thank the reviewer for the thorough revision of our manuscript. We agree with the reviewer's interpretation and extended, therefore, the discussion section of the manuscript.

A possible confusion (?) between "pheromone" and "volatile odorant" is found in the first paragraph, page 8. Of note, mouse parental behavior is shown to be dependent on the main olfactory function, but not on the accessary olfactory system conveying pheromone.

We agree with the reviewer. Therefore we replaced the word "pheromone" with "volatile odorant" in the text: *"In the next step, we performed an olfactory habituation/dishabituation test with a sequential presentation of pup odor, to determine whether Shank2*$^{-/-}$ *mice display a preference for the volatile odors of the pups"*

The method used for blood collection should be specified in the Method section.

We collected the blood for the oxytocin, prolactin, estradiol, and progesterone level measurement via cardiac puncture. We added this information now to the method section.

It wasn't clear to the reviewer why the described chemogenetic activation is limited to excitatory MPOA neurons as claimed in page 13, because both of the virus constructs used the hSyn promotor.

We agree with the reviewer. Therefore, we rephrased the result section of the manuscript in the context of the global activation of MPOA rather than excitatory MPOA neurons specifically.

1st Revision - Editorial Decision 20th Nov 2020

Dear Tobias,

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has now been seen by referees #1 and 3. As you can see from the comments below they both appreciate the introduced revisions. Referee #3 has some good suggestions regarding the title that I would like you to consider.

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

- For the figure files please upload high resolution figures. Please also double check that the text is large enough to read in the figures. Like some of the histograms are difficult to read. If you find the figures too crowded OK to add more or use expanded view figures https://www.embopress.org/page/journal/14602075/authorguide#expandedview

- You can only have 5 keywords at the moment you have 8

- We require a Data Availability section. This is the place to enter accession numbers etc. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories. Please place it after the Materials and methods and before Acknowledgements

- For the reference list - for articles with more than 10 authors the author list should be cut after 10 authors followed by et al.

- Will you make sure that all the funding info is entered in the submission system as well

- Double check that there are callouts to Fig 4B,C,E,F,G and Fig 6C

- The supplemental information file should be called Appendix and have a ToC. Please also add figure legends for the supplemental figures to the appendix. The figures should be labelled Appendix S1… Please also correct callout in text.

- Double check that the manuscript section order is correct.

- Please also provide us with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Wait to upload the revised version until you have received their comments.

That should be all

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 18th Feb 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have fully addressed all of review comments. I do not have any further comments.

Referee #3:

Comments for the revised version

In this revision, the authors attended very well with my original comments and revised the manuscript accordingly, except for the first comment on the term use.
Social bond is a very wide term, including pair-bond, mother-infant mutual bonding, and bonding between any individuals. On the other hand, this study examined only maternal behaviors toward pups. This is the reason why I think the title of this paper "Activation of the medial preoptic area ameliorates loss of social bonding in Shank2 -/- ASD mice" is overstatement. To be precise, the title should be changed to;
"Activation of the medial preoptic area ameliorates loss of maternal behavior in Shank2 -/- ASD mice"
Or at least
"Activation of the medial preoptic area ameliorates loss of maternal social bonding toward pups in Shank2 -/- ASD mice"

Otherwise, the authors should demonstrate that activation of the medial preoptic area also ameliorates loss of other social bonding behaviors in Shank2 -/- mice.

Response to reviews on manuscript EMBO: "Activation of the medial preoptic area ameliorates loss of social bonding in Shank2-/- ASD mice" by Grabrucker et al.

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has now been seen by referees #1 and 3. As you can see from the comments below they both appreciate the introduced revisions. Referee #3 has some good suggestions regarding the title that I would like you to consider.

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

a) For the figure files please upload high resolution figures. Please also double check that the text is large enough to read in the figures. Like some of the histograms are difficult to read. If you find the figures too crowded OK to add more or use expanded view figures

https://www.embopress.org/page/journal/14602075/authorguide#expandedview

We have created 600dpi tiff files and increased font sizes according to the figure guidelines.

b) You can only have 5 keywords at the moment you have 8

This has been corrected now.

c) We require a Data Availability section. This is the place to enter accession numbers etc. As far as I can see no data is generated that needs to be deposited in a database. If this is correct please state: This study includes no data deposited in external repositories. Please place it after the Materials and methods and before Acknowledgements

The sentence: "This study includes no data deposited in external repositories" has been added before the acknowledgment section of the manuscript now.

d) For the reference list for articles with more than 10 authors the author list should be cut after 10 authors followed by et al.

This has been corrected now. Further, the references have been formatted according to the manuscript's guidelines.

e) Will you make sure that all the funding info is entered in the submission system

as well

We have entered all necessary information now.

f) Double check that there are callouts to Fig 4B,C,E,F,G and Fig 6C

We have now made sure all figures are called out in the text of the manuscript.

g) The supplemental information file should be called Appendix and have a ToC. Please also add figure legends for the supplemental figures to the appendix. The figures should be labelled Appendix S1... Please also correct callout in text.

This has been corrected. We have selected 5 Figures, as per formatting guidelines, to be shown as "expanded view" and created a word file "Appendix" that shows the remaining supplementary information. The figures have been renamed accordingly in the text of the manuscript.

h) Double check that the manuscript section order is correct.

We made sure the sequence of the sections is correct now.

i) Please also provide us with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

Global deletion of SHANK2, a post-synaptic scaffold protein at excitatory synapses has been reported to cause autism-like behavior in mice. Shank2$^{-/-}$ mice are incapable of initiating social bonding towards pups.

- The neuronal mechanism that may explain the failure to develop this social relationship can be attributed to impaired glutamatergic signaling in a brain circuit (including the medial preoptic area (MPOA)) regulating social attachment. The behavioral alterations seem to be independent of hormonal influences, and cognitive and sensory deficits.
- Chemogenetic activation of MPOA neurons rescues the loss of social bonding in *Shank2*$^{-/-}$ mice.
- The MPOA might be a novel therapeutic target area to influence social behavior in ASD.

j) I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Wait to upload the revised version until you have received their comments.

We have based a revision on the version that had the pre-publication checks done and addressed all further comments in the file.

FURTHER Points: Instructions for preparing your revised manuscript:

a) Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

We checked again and believe that title and abstract are adequate. The title was changed based on the suggestions of the reviewer #3.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen: https://bit.ly/EMBOPressFigurePreparationGuideline

We have edited our figures to ensure they are in line with EMBO's figure guidelines.

Response to the Referees

Referee #1:

The authors have fully addressed all of review comments. I do not have any further comments.

We thank the referee for the second evaluation of our manuscript.

Referee#3:

Comments for the revised version

Social bond is a very wide term, including pair-bond, mother-infant mutual bonding, and bonding between any individuals. On the other hand, this study examined only maternal behaviors toward pups. This is the reason why I think the title of this paper "Activation of the medial preoptic area ameliorates loss of social bonding in Shank2 -/- ASD mice" is overstatement. To be precise, the title should be changed to;

"Activation of the medial preoptic area ameliorates loss of maternal behavior in Shank2 -/- ASD mice"

Or at least

"Activation of the medial preoptic area ameliorates loss of maternal social bonding toward pups in Shank2 -/- ASD mice"

Otherwise, the authors should demonstrate that activation of the medial preoptic area also ameliorates loss of other social bonding behaviors in Shank2 -/- mice.

We thank the referee for the second evaluation of our manuscript. We agree that the suggested title of the referee would be more appropriate. Therefore, we changed the title as suggested to: “Activation of the medial preoptic area (MPOA) ameliorates loss of maternal behavior in a *Shank2* mouse model for autism”.

2nd Revision - Editorial Decision 16th Dec 2020

Dear Tobias,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a close look at it and I appreciate the introduced changes.

I am therefor very pleased to accept the manuscript for publication here.

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Prof. Dr. Tobias M. Boeckers
Journal submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2019-104267R

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple 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.

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

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

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size calculation were based on relevant published literature with similar experimental design and on our previous published research.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size calculation were based on relevant published literature with similar experimental design (Guo et al., 2019).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusion was performed.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	no subjective assessment of the data was made.
For animal studies, include a statement about randomization even if no randomization was used.	Selection of mice was based on genotype and sex. All other factors were randomized.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Samples and groups were assigned randomly and treated equally. If possible, experiments were conducted in a blinded manner.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Due to the obvious behavioral phenotype of the knockout mice, blinding was not always possible.
5. For every figure, are statistical tests justified as appropriate?	Yes, normal distribution was determined by Shapiro-Wilk test. For normally distributed data, single comparisons were tested using independent Student's t-test. Non-parametric data were examined using the Mann-Whitney U-Test. Experiments with more than two groups were subjected to a one-way ANOVA if normally distributed and compared using Bonferroni's post hoc test. Multiple comparisons of nonparametric data were made by a Kruskal-Wallis analysis followed by Dunn's post hoc test. Experiments with two between factors (treatment, genotype) were analyzed using a two-way ANOVA, to determine genotype and treatment effect or interaction between both factors. Experiments with one "between-subjects" factor (genotype) and one "within-subjects" factor (trial) were analyzed using a two-way mixed ANOVA. Statistical tests are mentioned in the figure legend.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution of the data was determined by Shapiro-Wilk test.

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Is there an estimate of variation within each group of data?	We have calculated variance, standard deviation as well as standard error of the mean for each dataset. Data are depicted as mean with standard error of the mean (SEM) in the manuscript.
Is the variance similar between the groups that are being statistically compared?	Not all variances were similar in the study (statistical tests were used to account for dissimilar variance).

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The catalog number and dilution factor of the primary and secondary antibodies are all listed in the material and method section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used in the study.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	species: Mus musclus, strain: C57BL/6J, gender: female/male, genetic modification: the generation of the knock out mouse line has been reported previously (Schmeisser et al, 2012). All mice were housed in mixed-genotype groups of 3-4 per cage and randomly selected for behavioral or biochemical experiments. All mice were bred and housed according to standard laboratory conditions and provided with food and water available ad libitum. The housing room was maintained at 22°C, with lights automatically turned on/off in a 12 h rhythm (lights on at 7 am).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments were performed in compliance with instituitonal guidlines and approved by Regierungspräsidium Tübingen.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We comply with the ARRIVE guidlines.

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
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21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No