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An inter-dimer allosteric switch controls NMDA receptor activity

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Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   16th Jul 2018

Thank you for submitting your study to The EMBO Journal. Your study has now been seen by two referees. A third referee who had agreed to review the study has unfortunately not returned his/her referee report and at this stage I don't think we will receive it. I will therefore take the decision based upon the two referee reports at hand.

As you can see from the reports below, both referees appreciate the reported findings and the methodology used. However, they also both raise some important points that should be addressed in a revised version. Given the input received, I would like to invite you to submit a revised a version that takes into consideration the concerns raised. I should add that it is EMBO Journal policy to allow only a single major round of revision, and that it is therefore important to address the concerns raised at this stage.

REFEREE REPORTS:

Referee #2:

This is an interesting paper that uses mutagenesis to support modelling of NMDA receptor domain movements that are hypothesized to represent steps on the reaction path between agonist binding and channel opening. The work is important because there remains poor understanding despite much structural and functional work about how glutamate receptors operate. The authors use conventional crosslinking to presumably prevent the motions that are modelled to be involved in controlling receptor gating, providing supporting data for their interpretations. The functional effects of redox modulation of Cys pairs was supported by biochemical analysis of crosslinking. The crosslinks that prevent or trap hypothesized rolling motions also influenced allosteric modulation, and had modest

effects on glutamate and glycine EC50 (2-4 fold changes). The paper provides the research community with valuable new data from clever experiments, and this will be influential in guiding new experimentation about how these receptors operate. I have only a few comments to consider.

1. My main critique is with the caveat that mutations at key regions might alter function in a manner that is unrelated to the rolling motion. Key crosslinked residues could, simply because they are important in unforeseen ways, reduce or enhance response by acting on critical regions of the receptor, not because the rolling motion that is hypothesized actually takes place. While I agree the authors' conclusions are appropriate and model is reasonable, some recognition of the risk of mutating and crosslinking protein-protein interfaces would strengthen the paper by providing a measured, scholarly consideration of the potential pitfalls of this approach. As it is, no caveats are discussed, giving the impression no caution is needed in the interpretation of modelling and mutagenesis data.

2. A second criticism is that no information is provided to show how locking the receptor in the "rolled" state affects the time course of the macroscopic current. This seems straight forward, and could provide a wealth of information. Does locking the receptor alter the rise time, deactivation, or desensitization?

3. It would be worth considering how modulators that interact with the ligand binding dimer interface (TCN-201, GNE compounds) might be affected by crosslinking given the proposed mechanism. Straightforward experiments might be informative and speak to the effects of the "rolling" motion on modulators that act outside the amino terminal domain, which are therapeutically interesting.

Minor points

4. Figure 1B-Are the DTE treated traces all on the same scale? Perhaps state this in the legend.

5. Page 8, first sentence of the last paragraph. It is not clear which model was fitted-the rolled, locked model with Cys-Cys between GluN2B L795 and GLuN1 E698?

Referee #3:

In this paper Esmenjaud et al., study allosteric coupling between the NMDAR extracellular domain layers and between ABD dimers using a combination of electrophysiology, biochemistry and simulations. Using current NMDAR structures as guides for cysteine mutagenesis they highlight the importance between coupling of NTD and ABD layers and identify a novel interaction between ABD dimers that affects gating and is modulated by conformations in the NTD layer. This is a nice and solid piece of work that shines new light on NMDAR gating mechanics and could provide a substrate for future structural work. The paper is well written and the experiments solid and well documented.

detailed comments:
As the authors point out, the GluN1 E698C single mutant (ie in combination with GluN2B WT) results in crosslinks on gels, presumably between the GluN1 subunits (Fig. 1d; interface 5). This particular combination also seems to contribute functional effects via the NTD such as Zn IC50 and the effect of spermine (Suppl. table 3) as well as agonist EC50s'. Although these effects are smaller than the ones seen for the double mutant combi., GluN1 E698C/GluN2B L795C, the authors should still discuss this aspect more, particularly in context of their structural model/model schematics.

With regard to the simulations (Fig. 4), I was wondering how reproducible the trajectory, obtained from MODFIT, was. Specifically, do the 3 steps described always take place in the same order 1->3, or do they also obtain runs where a different set of modes is initially accepted but the trajectory is different? In short, it would be useful to state how much variability there is.

It is mentioned in the intro and discussion that in AMPAR the NTD layer floats above the LBD (or ABD) layer, whereas the two layers are more closely connected in NMDARs. Although this

'floating' AMPAR NTD holds for current GluA2 homomer structures it remains to be seen for nonGluA2 AMPARs, where NTD dimers will associate via sequence-different interfaces. Indeed, in GluA2/A3 receptors a more compact, NMDAR-like, organisation could be trapped in a recent structure (Herguedas et al. 2016) and was seen by normal mode analysis (Dutta et al., 2015) - this should be acknowledged.

on p. 7, para 2 in the middle, the authors refer to Fig 2b and lower down to Fig 2c but presumably mean Fig 3.

1st Revision - authors' response   10th Sep 2018

EMBOJ-2018-99894
Revised manuscript by Esmenjaud *et al.*
Reply to referees

We thank the two reviewers for their insightful and constructive comments on our manuscript. According to these comments, we have made several modifications to our work. We have included new experimental data and clarified several points at various locations in the text. We believe that altogether these revisions lead to an improved manuscript.

<u>**Referee #2**</u>

I have only a few comments to consider.

1. My main critique is with the caveat that mutations at key regions might alter function in a manner that is unrelated to the rolling motion. Key crosslinked residues could, simply because they are important in unforeseen ways, reduce or enhance response by acting on critical regions of the receptor, not because the rolling motion that is hypothesized actually takes place. While I agree the authors' conclusions are appropriate and model is reasonable, some recognition of the risk of mutating and crosslinking protein-protein interfaces would strengthen the paper by providing a measured, scholarly consideration of the potential pitfalls of this approach. As it is, no caveats are discussed, giving the impression no caution is needed in the interpretation of modelling and mutagenesis data.

We now include a discussion about the risk of protein-protein interface cross-linking, thus leading to a more cautious presentation of the approach and conclusions (Discussion p13, 2nd paragraph).

2. A second criticism is that no information is provided to show how locking the receptor in the "rolled" state affects the time course of the macroscopic current. This seems straight forward, and could provide a wealth of information. Does locking the receptor alter the rise time, deactivation, or desensitization?

As requested, we have performed additional experiments to investigate how the rolled state affects receptor macroscopic kinetics. For that purpose, we measured whole-cell current responses of HEK293 cells expressing the mutant receptors locked in the 'rolled' state. We focused most particularly on glutamate deactivation kinetics, a parameter classically measured because of its critical importance in synaptic physiology, and desensitization. These new experimental results have all been included and illustrated in the revised manuscript (Results section p8 and new Appendix Figs S3 and S4).

We found glutamate deactivation of GluN2B rolled receptors to be slightly accelerated compared to WT receptors (i.e. faster off-relaxation; τ_{off} = 272 ± 47 ms [n=6] for CC receptors *vs* 414 ± 69 for WT receptors; P=0.003), in very good agreement with the modest (2-fold) increase in glutamate EC_{50} previously described. A change in glutamate deactivation kinetics paralleled to a change in glutamate apparent affinity was also observed at rolled GluN2A receptors (Appendix Fig S4D). Regarding desensitization, our recordings clearly show that

'rolled' receptors can still enter into desensitized states. However, we found that 'rolled' receptors exhibit less desensitization than their WT counterparts (applying both for GluN2A and GluN2B receptors). Effects are of moderate amplitude (see Appendix Figs S3E and S4E): for GluN2B receptors, I_{ss}/I_{peak}= 0.81 ± 0.1 [n=5] for CC receptors *vs* 0.59 ± 0.14 [n=5] for WT; for GluN2A receptors, I_{ss}/I_{peak}= 0.69 ± 0.27 [n=5] for CC receptors *vs* 0.32 ± 0.23 [n=6] for WT. Desensitized states usually show higher agonist sensitivity than resting and active states. Therefore, that receptors locked in the 'rolled' state display reduced desensitization is interesting since it is provides an additional potential contributing factor for why 'rolled' receptors do not show enhanced agonist sensitivity (as expected from their greatly enhanced gating efficacy). This is briefly discussed in the revised manuscript (p15).

3. It would be worth considering how modulators that interact with the ligand binding dimer interface (TCN-201, GNE compounds) might be affected by crosslinking given the proposed mechanism. Straightforward experiments might be informative and speak to the effects of the "rolling" motion on modulators that act outside the amino terminal domain, which are therapeutically interesting.

We have performed new experiments to assess the impact of ABD inter-dimer crosslinking on the sensitivity to TCN-201, a GluN2A-negative allosteric modulator that binds the ABD intra-dimer interface (Hansen et al., 2012; Yi et al., 2016). Interestingly, we found that TCN-201 (1 µM) was equally competent to inhibit WT and cross-linked receptors (see new Appendix Fig 4B; note that glycine concentrations were adjusted to insure similar glycine site occupancy between the two receptor types). This is in striking contrast with what we observed with NTD modulators (drastic reduction in sensitivity). We believe that these results make good sense with what is known about TCN-201 action - local perturbations at the level of the ABD *intra-dimer* interface (Yi et al., 2016) - and our proposed transduction mechanism of long-range interlayer coupling through *inter-dimer* ABD rolling-unrolling motions. As stated in the Discussion, 'rolling' provides an upstream control mechanism on the ABD-TMD gating core.

The TCN-201 data are included, discussed and illustrated in the revised manuscript (Results section p8, new Appendix Fig 4B and Discussion section p14). Two new references have also been added to the reference list (Hansen et al., Journal of Neuroscience 2012 and Yi et al., Neuron 2016).

Minor points

4. Figure 1B-Are the DTE treated traces all on the same scale? Perhaps state this in the legend.

The DTE-treated traces are not all on the same current scale but have been normalized to the height of the maximal post-DTE response for WT receptors. The purpose is to highlight the difference in current intensity before and after redox (DTE) treatment between the different mutants. Given the large variability in NMDAR-mediated current amplitude from one oocyte to the other typically observed following cDNA injections, normalizing for absolute current intensities would make the comparison difficult. We now explicitly state how traces were normalized in the revised legend (Figure 1B).

The model used for the fitting is not based on the receptor with the introduced cross-linked but on the 'wild-type' (C-terminal lacking) receptor in complex with glutamate, glycine and the GluN2B antagonist ifenprodil (i.e. in an inhibited state). This is clearly stated in the Methods section. We also mention it in the Results section a few lines above the mentioned sentence, but it may have lacked clarity. To remove any ambiguity, we have added 'wild-type' to the previous paragraph and modified the sentence to: 'When fitting our full-length model of the inhibited state into the TMD-missing 'active' state EM map…' (page 8).

Referee #3

Detailed comments:

As the authors point out, the GluN1 E698C single mutant (ie in combination with GluN2B WT) results in crosslinks on gels, presumably between the GluN1 subunits (Fig. 1d; interface 5). This particular combination also seems to contribute functional effects via the NTD such as Zn IC50 and the effect of spermine (Suppl. table 3) as well as agonist EC50s'. Although these effects are smaller than the ones seen for the double mutant combi., GluN1 E698C/GluN2B L795C, the authors should still discuss this aspect more, particularly in context of their structural model/model schematics.

As we acknowledge in the paper, and in line with a previous study from our lab (Riou et al., 2013), co-expressing GluN1-E698C single mutant subunit with a wild-type GluN2 subunit can lead to the formation of cross-linked GluN1 subunits. As shown in the current study (Figures 2C and S3), this cross-link alone impacts GluN1/GluN2B receptor activity, although not all properties are affected (decrease in zinc, ifenprodil, spermine and glycine sensitivity; no effect on pH and glutamate sensitivity and on channel maximal Po as assessed by MK-801 inhibition kinetics). Clearly, the possibility of GluN1 homodimer formation is an issue when interpreting data obtained from double cysteine mutant receptors (GluN1-E698C/GluN2B-L795C). However, several line of evidence indicates that homodimer GluN1 cross-links are unlikely to contribute significantly to the (striking) phenotype of the double cysteine mutant:

- Double mutant cysteine receptors show a greatly enhanced Po (not far to unity), yet single GluN1-E698C mutant receptors show no (or little) Po effect. If both receptor cross-liks (homo and hetero) were present in significant amount, Po should lie in between wild-type and unity. What is observed is a Po close to unity, strongly supporting a large dominance in expression (and function) of hetero mutants.

- Similarly, the pH sensitivity curve of the double cysteine-mutant is strongly shifted and shows no sign of 'altered' Hill coefficient. Because the single GluN1-E698C mutant shows no modification of pH sensitivity (compared to WT receptors), a mixture of receptor populations would result in biphasic curves and/or altered DRC slopes. Again, this is not what is observed, strongly supporting that the measured currents are mostly (if not exclusively) carried by hetero mutants.

- Western blots, as indicated by band intensities with the anti-GluN1 antibody (Figure 1D), provide independent evidence that when GluN1-C and GluN2-C are co-expressed, the vast majority of cross-linked subunits are between GluN1 and GluN2 and not between two GluN1 subunits (hetero >> homo).

We believe that, altogether, these results, make it (very) unlikely that homo GluN1-C cross-links are interfering with observed phenotype of the double cysteine mutant receptor. We infer that when both GluN1-C and GluN2-C subunits are co-expressed, hetero cross-links are greatly favored compared to homo cross-links.

In the revised manuscript, we now specifically mention and discuss results obtained with the GluN1-E698C single mutant and why there are unlikely to interfere with the observed phenotypes of the double mutants (Results section, Page 7).

With regard to the simulations (Fig. 4), I was wondering how reproducible the trajectory, obtained from MODFIT, was. Specifically, do the 3 steps described always take place in the same order 1->3, or do they also obtain runs where a different set of modes is initially accepted but the trajectory is different? In short, it would be useful to state how much variability there is.

The question of robustness and variability of our iModFit simulations is indeed an important one. As detailed below, several piece of evidence point to a robust situation. First, as mentioned in the Methods section of the initial submission, we already had conducted a series of control simulations with the exact same starting 3D model and final experimental EM data but with different EM map threshold (<cutoff> parameter), different range of modes (-n option), or fixed secondary elements dihedral (-S option). In each case, we obtained (very) comparable trajectories than the one with default program options presented in the manuscript. Importantly, all of them show the typical rolling motion and the pore dilation. Moreover, they all pass through the experimentally determined non-active state (pdb 5FXI; agonist bound, no antagonist), even though this structure is not used as input in the simulations. Comparisons of rmsd, which were not illustrated in the first version of our manuscript, are now presented in the new Appendix Figure S6B-D.

In addition, one source of variation in iModFit simulations in the random choice of modes made at each step. In order to test the extent of variation introduced by this randomization, we repeated 21 times the simulations of the trajectory presented in Figure 4A. These replicates allowed us to calculate a mean rmsd (± sd) of the trajectory. We now provide this information as a plot in the new Appendix Figure S6A. It clearly shows that each of the 21 replicates follows the same trajectory, with marginal differences. Careful visualization of each replicate also reveals that the trajectories always show the three steps initialed described, with the typical NTD compaction, ABD rolling motion and pore dilation towards the end of the run. In conclusion, our iModFit trajectories appear robust. In the revised manuscript, accompanying the new Appendix Figure S6, we have extended the text, both in the Results (p 9) and Methods (p 20) sections.

It is mentioned in the intro and discussion that in AMPAR the NTD layer floats above the LBD (or ABD) layer, whereas the two layers are more closely connected in NMDARs. Although this 'floating' AMPAR NTD holds for current GluA2 homomer structures it remains to be seen for nonGluA2 AMPARs, where NTD dimers will associate via sequence-different interfaces. Indeed, in GluA2/A3 receptors a more compact, NMDAR-like, organisation could be trapped in a recent structure (Herguedas et al. 2016) and was seen by normal mode analysis (Dutta et al., 2015) - this should be acknowledged.

We now refer to the heteromeric GluA2/A3 work both in the Introduction (p 4) and Discussion (p13) and have added the corresponding references. We also specifically comment on the possibility that GluNA2/A3 receptors may adopt more NMDAR-like compact structure raising the possibility of functional allosteric interactions between the NTD and ABD layers (see Discussion p13).

on p. 7, para 2 in the middle, the authors refer to Fig 2b and lower down to Fig 2c but presumably mean Fig 3.

Corrected. Thank you for spotting this.

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by referee # 3 and as you can see from the comments below the referee appreciates the introduced changes. I am therefore very happy to accept the manuscript for publication in the EMBO Journal.

Before sending you the formal acceptance letter there are just a few things to sort out. You can use the link below to upload the files.

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- We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Could you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- It would also be good if you could provide me with a summary figure that I can place in the synopsis. The size should be 550 wide by 400 high (pixels).

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That should be all. As soon as I receive the revised version I will formally accepted the manuscript. Congratulations on a nice study.

REFEREE REPORTS:

Referee #3:

The authors have addressed all my concerns, I recommend publication of this work

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Corresponding Author Name: PAOLETTI Pierre
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-99894

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.

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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
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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).
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➔ 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;
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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)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to pre-determine smaple size. Sample size was chosen for the various experiments according to typical numbers of observations in the respective field (e;g. cellular electrophysiology; n numbers between five and a few tens)
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2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusion criteria was used
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.	Randomization was not performed, but mutant receptors were systematically compared/processed in parallel to control wild-type (WT) receptors. Moreover, data of experiments were systematically double-checked by another observer and contributor of the study.
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	No blinding was performed. Yet, mutant receptors were systematically compared/processed in parallel to control wild-type (WT) receptors. Moreover, data of experiments were systematically double-checked by another observer and contributor of the study.
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5. For every figure, are statistical tests justified as appropriate?	Yes, statistical tests are identified in figure legends. When only two groups were compared, Student's t-test was used. When n>2 groups were compared one-way ANOVA was used.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution is assumed (i.e. value of interest exhibits a bell-curve distribution function) as classicaly done for study of biophysical parameters of ion channels and receptors.
Is there an estimate of variation within each group of data?	Yes, standard deviation of the mean (SD) is systematically reported (see figure legends)
Is the variance similar between the groups that are being statistically compared?	Yes, variation between groups was similar.

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).	Details on antibodies, including clone number or catalog number, are specified for each antibody used (see Methods 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.	As mentioned in the Methods section, HEK cells were obtained from ATCC Inc.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Raw and analysed data are stored on dedicated servers of the main participating institution (IBENS, ENS, Paris, France).
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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.	NA

G- Dual use research of concern

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