EMBO reports

Disrupted Hypothalamic CRH Neuron Responsiveness Contributes to Diet-induced Obesity

Canjun Zhu, Yuanzhong Xu, Zhiying Jiang, Jin Bin Tian, Ryan M. Cassidy, Zhaolin Cai, Gang Shu, Yong Xu, Mingshan Xue, Benjamin R. Arenkiel, Qingyan Jiang and Qingchun Tong

Review timeline:

Submission date:	3 September 2019
Editorial Decision:	7 November 2019
Revision received:	9 March 2020
Editorial Decision:	17 April 2020
Revision received:	19 April 2020
Accepted:	24 April 2020

Editor: Esther Schnapp

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 7 November 2019

Thank you for the submission of your manuscript to EMBO reports. We have now received the enclosed reports on it.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also have several suggestions for how the study should be strengthened for publication here. I think all comments are reasonable and should be addressed. Please let me know if you disagree, and we can discuss this further.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-

values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) Your manuscript contains statistics and error bars based on n=2 or on technical replicates. Please use scatter blots in these cases. No statistics can be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

8) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from

which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:
The authors investigate the role of hypothalamic CRH neurons in diet-induced obesity (DIO). They use a combination of gold-standard assessments and experimental tools to assess the effects of chronic activation or inhibition fo CRH neurons on appetite and energy metabolism in high-fat fed mice.
Technically, the paper is of very high quality but the hypothesis tested by the authors is not clear. This should be clarified in the introduction.
They mention the role of CRH diurnal activity patterns but do not directly test how these patterns can promote hyperphagia together with a suppression of energy expenditure in DIO. Why is hyperphagia not increasing energy expenditure here?
The interpretation that the obesity phenotype is caused by the loss of nycthemeral regulation of the HPA axis lacks experimental support. If this is the main conceptual advance of the study, this needs to be demonstrated directly. In addition, a more in depth characterisation of the coordination of feeding and energy expenditure under these conditions is required to understand the cause of weight gain. The authors should show mean day time and mean night time energy expenditure and food intake in additional to what's shown.

What is causing the blunted responsiveness of CRH neurons in the fast-refeed paradigm or other stimuli that activate the CRH axis? At a minimum, possible contributing factors should be discussed in the discussion.

Referee #2:
The idea of linking CRH PVN neurons to obesity is novel and interesting, however, additional data is needed to support hypothesis.

Major Comments
1. In Figure 2 the representative trace shows that NachBac side presents a lower frequency with increased after-potential hyperpolarization which, in general, characterize inhibition of the given neuron. How is it considered a high activity clamping?
How is it possible that the activity of the CRH neurons are higher if they show a hyperpolarized membrane resting potential in comparison to the control animals?
2. The authors claim "Chronic stress induces a sustained increase in the activity of PVH CRH neurons [37], which may mimic the effect induced by NachBac in clamping the neuron activity at high levels. Thus, disrupted neuron responsiveness may explain obesity development induced by

chronic stressors (e.g. social stress)" but the animals with high-activity clamped CRH neurons did not show obesity, only when in an HFD regimen.
3. How would NachBac injected animals mimic social stress conditions? Data on behavior and corticosterone levels should be shown.
4. When discussing "diurnal patterns" additional parameters need to be measured? Activity levels and 24-hour corticosterone level profile would be better support data for the authors' assumptions.
5. If disrupted hypothalamic CRH neuron responsiveness contributes to DIO, why animals with their CRH neuron activity clamped at low or high levels did not show changes in their metabolic stage? It seems that the lack of CRH neurons responsiveness to a change in the nutritional environment is a consequence of DIO and does not present a causality component to obesity.
6. An experiment showing CRH PHV rescue of responsiveness should be performed in an attempt to link the lack of responsiveness to obesity.

Minor Comments
1. The entire manuscript should be carefully revised as it presents a high incidence of typos, divergent nomenclature viruses and citations standardized throughout the document.
2. CRH-Cre mouse is a well-established model, and the authors have to provide citations and background information.
3. Would be appropriate if the authors verify the co-localization of tdTom and CRH in the CRH-Cre::Ai9 reporter mouse.
4. Chow and HFD ad lib animals should be demonstrated in Figure 1 alongside with fasted and refed mice.
5. In Figure 2 there is some contradictory information. The authors first describe it as bilateral injection of Flex-EGFP-P2A-mNachBac and then claim that they used the contralateral side as control (second paragraph, page 5).
6. The Corticosterone basal level of the animals that were treated with DEX should be shown as the animals were adapted in the experimental chamber for 15 minutes prior to the recording, this change would be enough to elicitate stress response in the animals.

Referee #3:
In this manuscript, Zhu and colleagues explore the effect of a HFD on PVN CRH neurons. They show that HFD blunts the response of the CRH neurons to a stressor and to Dex. They then attempt to mimic the HFD condition by clamping the electrical activity of these neurons using genetic tools, and show that whether clamped at a higher or lower functional level, it rendered the neuron unable to respond to stimuli, and resulted in hypersensitivity to HFD in terms of weight gain. Thus they conclude that it isn't necessarily the absolute function of the CRH neurons that is important, rather its ability to respond to a stimuli.

I do have some issues that I would like addressed

1) There are a lot of moving parts to this study, and I think that the investigators are somewhere from showing that the artificial clamping of neuronal function in anyway mimics a HFD. The clamping of the neurons may simply have turned out to be a sophisticated way of 'knocking out' the function of the cells, therefore resulting in a phenotype. The response of the neurons to a high-fat diet is far more subtle. I understand why the authors have interpreted the data as such, but I think they should be more circumspect in their reporting.

2) Have the investigators measured what happens to CRH, either at the transcript of protein level, in response to all of the perturbations? HFD or the clamping? I think this would add weight to the argument that this could play a potential role in stress induced weight gain.

3) This is minor, but there are many typos and grammatical errors sprinkled throughout. This manuscript will need careful copyediting.

We would like to thank all reviewers for their insightful comments, to which we have provided point-to-point responses. We hope that the reviewers concur with us that the new version has been significantly improved and can be accepted in EMBO Reports.

Referee #1:

1) Technically, the paper is of very high quality but the hypothesis tested by the authors is not clear. This should be clarified in the introduction.

Response: We have clarified our hypothesis in the introduction.

2) They mention the role of CRH diurnal activity patterns but do not directly test how these patterns can promote hyperphagia together with a suppression of energy expenditure in DIO. Why is hyperphagia not increasing energy expenditure here?

Response: The reviewer asks an interesting question on coupling between feeding and energy expenditure: increased feeding should come with an increased energy expenditure, which is normally observed in mice. However, this concept largely implicates static regulation of feeding and energy expenditure. Here the main phenotype is the alteration in patterns of feeding, energy expenditure and locomotion. For example, in our NachBac model, compared to controls, these mice eat more feeding but spend less energy during light periods (Response Figure 3A, i.e. Figure R3A versus Figure R3C below). The same is true for Kir2.1 mice (Figure R4A versus Figure R4C below). These results suggest that both models exhibit higher feeding efficiency during day periods, which may contribute to DIO. To avoid confusion and clarify this issue, we've provided discussion on this point in Results and Discussion. The new data have been added as new Expended View Figure EV3 and EV5.

3) The interpretation that the obesity phenotype is caused by the loss of nycthemeral regulation of the HPA axis lacks experimental support. If this is the main conceptual advance of the study, this needs to be demonstrated directly. In addition, a more in depth characterisation of the coordination of feeding and energy expenditure under these conditions is required to understand the cause of weight gain. The authors should show mean day time and mean night time energy expenditure and food intake in additional to what's shown.

Response: We are sorry that our description of the hypothesis is not clear, causing some confusion. Our central hypothesis is that disruption of PVH CRH neuron responsiveness contributes to HFD-induced obesity. PVH CRH neurons are known to project a number of downstream brain regions and the HPA axis represents one arm of CRH neuron function. Although HPA activity can be used as a useful readout to verify our model, the goal of this study is not focusing specifically on HPA axis per se.

We agree with the reviewer that we didn't measure directly PVH CRH neuron diurnal activity pattern, which is currently technical challenging to achieve. However, our data on disruption of CRH neuron responsiveness in contributing DIO is very compelling, as evidenced from both physiological c-Fos and in vivo acute responses to stress, as well as 2 complementary models with CRH neuron activity clamped at high or low levels, both resulting in a similar sensitivity to DIO with disrupted feeding/metabolism rhythms. We indeed provided speculation that disruption of CRH neuron responsiveness may lead to disrupted CRH neuron diurnal activity pattern, and we also provided new supporting data including Kir2.1 mice exhibited flattened corticosterone patterns and NachBac mice exhibited increased CRH expression during both day and night periods. Nevertheless, our core conceptual advance is that disrupted hypothalamic CRH neuron responsiveness contributes to diet-induced obesity". To avoid this confusion, we have provided a discussion point in the Results and Discussion.

According to the reviewer's comment, we have presented data on day and night periods in feeding, energy expenditure and locomotion as shown in Figure R3 and Figure R4 below. Consistent with lack of dynamic changes in CRH neurons, the mice exhibited reduced energy expenditure during dark time when energy expenditure is normally high, and increased feeding during day time when feeding is normally low. This new data set has been added as new Figure EV3 and EV5.

4) What is causing the blunted responsiveness of CRH neurons in the fast-refeed paradigm or other stimuli that activate the CRH axis? At a minimum, possible contributing factors should be discussed in the discussion.

Response: We would like to thank the reviewer for this interesting question. As a matter of fact, this question is one of our current investigations in the lab and our preliminary data suggest that HFD, while has no obvious effects on mEPSC frequency (Figure R1A, N means animal number and n means neuron number), at least reduces mEPSC amplitude in PVH CRH neurons (Figure R1B). This is consistent with a change in PVH CRH neurons by HFD. Since we are still investigating GABAergic inputs, this set of data is not yet complete. However, per the reviewer's suggestion, we have provided a discuss point on possible contributing factors in Results and Discussion.

Referee #2:

Major Comments

1. In Figure 2 the representative trace shows that NachBac side presents a lower frequency with increased after-potential hyperpolarization which, in general, characterize inhibition of the given neuron. How is it considered a high activity clamping? How is it possible that the activity of the CRH neurons are higher if they show a hyperpolarized membrane resting potential in comparison to the control animals?

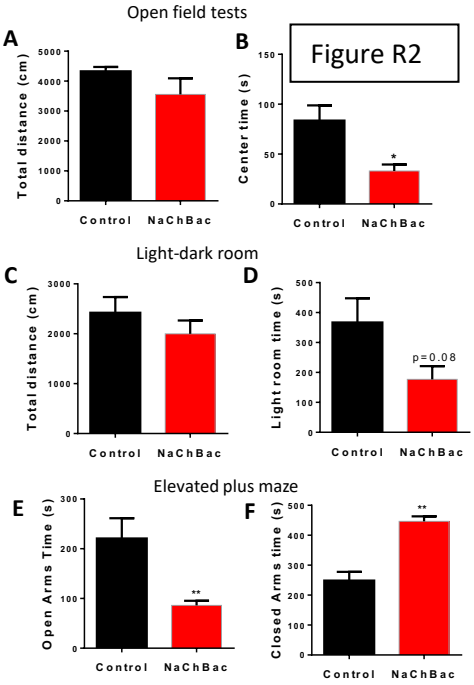
Response: We agree with the reviewer's statement on neuron hyperpolarization and neuron activity, which, however, is under the assumption that the neurons express the same types of sodium channels. In our case, these neurons express a type of sodium channel with much lower threshold for action potential (AP) firing and a very slow inactivation (as shown in inset in Fig. 2B), which means that even at a hyperpolarized membrane potential, NachBac-expressing neurons can still fire AP, and one NachBac-mediated AP allows more Na+ influx than hundreds of regular Na+ channel mediated AP. The more hyperpolarization of NachBac-expressing neurons may reflect a compensatory response to prevent over-excitation. In addition, the increased neuron activity with NachBac expression is also reflected by our c-Fos data (Figs. 2D-2F), and our new data on corticosterone and CRH levels (Figure EV2).

2. The authors claim "Chronic stress induces a sustained increase in the activity of PVH CRH neurons [37], which may mimic the effect induced by NachBac in clamping the neuron activity at high levels. Thus, disrupted neuron responsiveness may explain obesity development induced by chronic stressors (e.g. social stress)" but the animals with high-activity clamped CRH neurons did not show obesity, only when in an HFD regimen.

Response: We feel sorry that our presentation of body weight is not clear. As shown in Fig. 3A, NachBac mice exhibited increased body weight on chow diet at 6 weeks and 7 weeks after viral delivery. In order

to clarify this point and prevent confusion to readers, we have changed to individually label the significance for data pionts of each week in Fig. 3A.

3. How would NachBac injected animals mimic social stress conditions? Data on behavior and corticosterone levels should be shown.

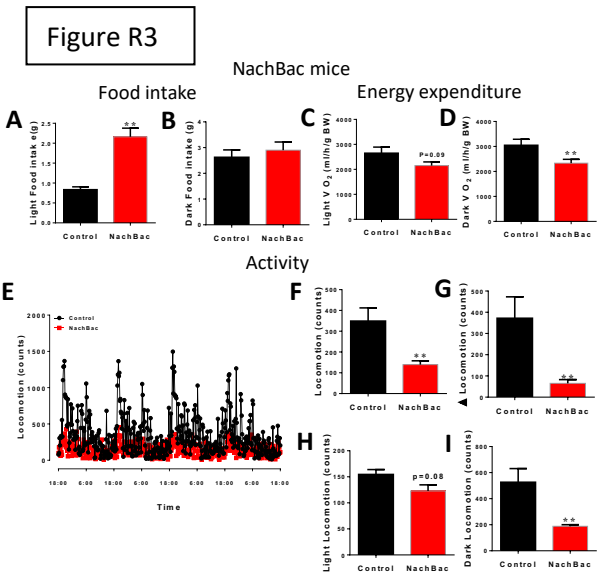


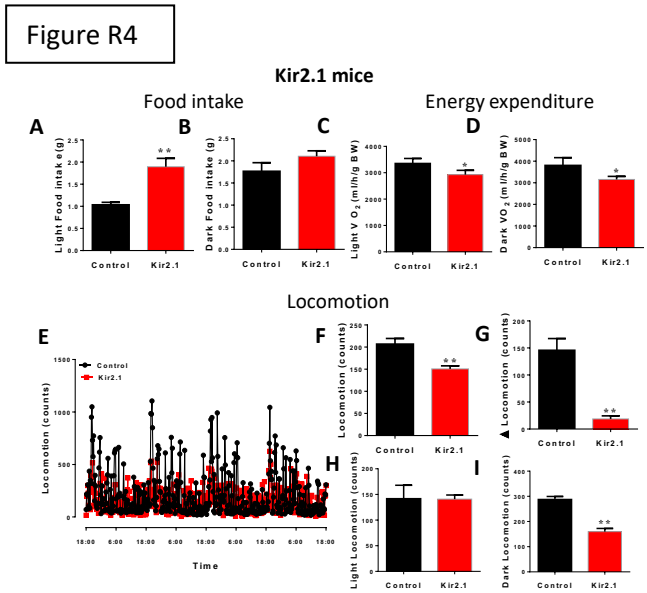
Response: We would like to thank the reviewer for this interesting and important question. Since social stress in rodents with a focus on CRH neurons is scarce, we are not sure how CRH neurons behave in social stress conditions. However, to address the reviewer's inquiry, we nevertheless performed behavioral tests including open field (Figure R2A and R2B), light-dark room (Figure R2C and R2D) and elevated plus maze (Figure R2E and R2F). NachBac mice showed an increase anxiety in all 3 tests examined, suggesting chronic activation of PVH CRH neurons causes increased anxiety levels. This new data set has been presented in new Appendix Fig. 2.

Please see the hormone data in our response to your Comment 4 below.

4. When discussing "diurnal patterns" additional parameters need to be measured? Activity levels and 24-hour corticosterone level profile would be better support data for the authors' assumptions.

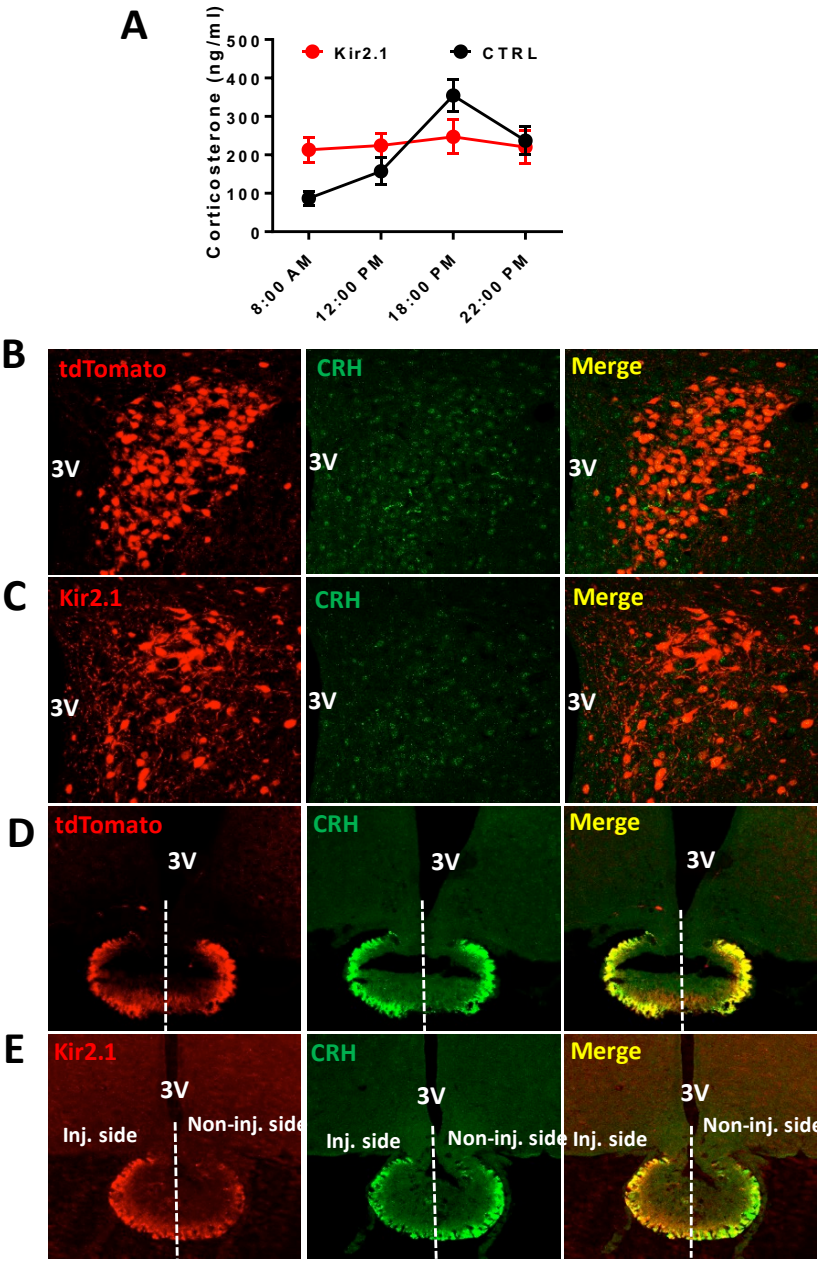
Response: We have now included 24hr activity level data as well as individual day and night time readings on feeding and energy expenditure for NachBac mice (Figure R3E-R3I below) and Kir2.1 mice (Figure R4E-R4I below). In both models, the movement is significantly reduced during dark periods. In addition, we also showed data for individual day and night feeding for NachBac (Figure R3A-3B), and Kir2.1 mice (Figure R4A-R4B) and energy expenditure for NachBac (Figure R3C and R3D), and Kir2.1 mice (Figure R4C and R4D). Both Figures have been added to the manuscript as new Figure EV 3 (NachBach) and new Figure EV5 (Kir2.1).



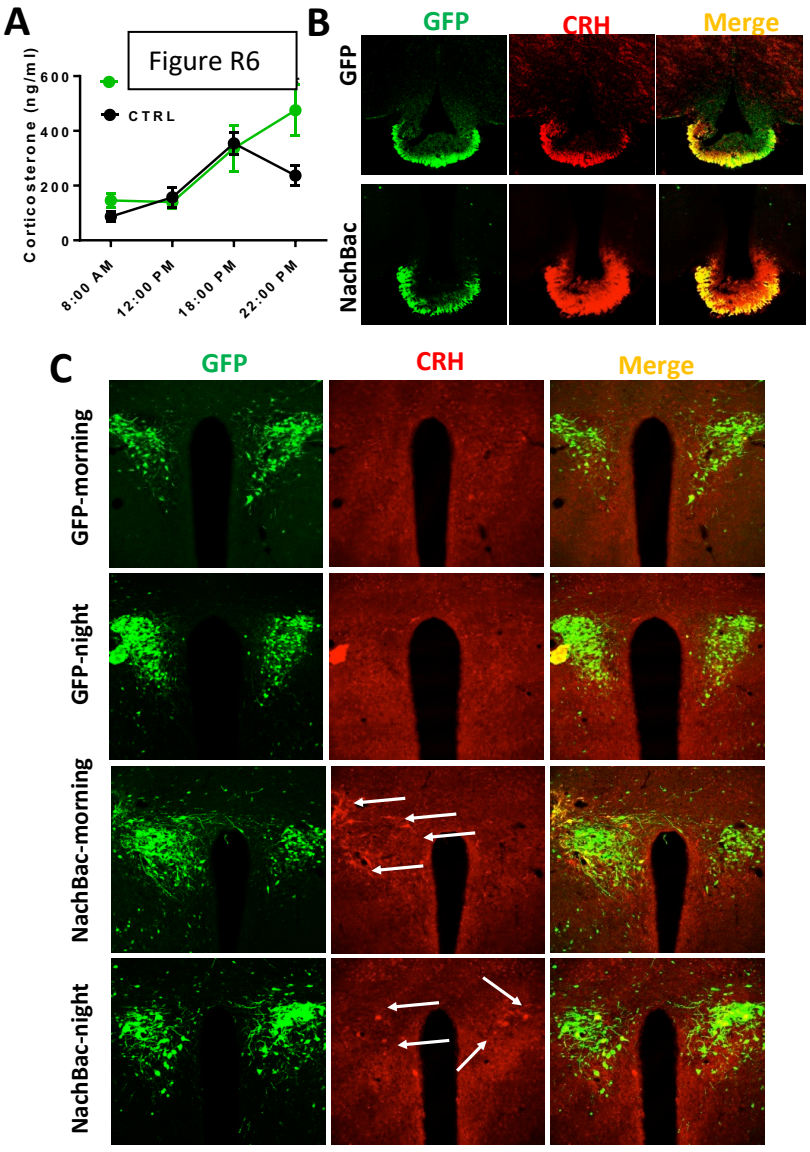


For 24hr corticosterone levels, we have also measured in Kir2.1 mice (Figure R5A) and also CRH expression in response to Kir2.1 expression in the PVH (Figure. R5B and R5C) and median eminence (ME, Figure R5D and R5E). As expected, control mice exhibited higher corticosterone levels during night time and consistent with the Kir2.1 effect on inhibiting CRH neurons, the diurnal pattern of corticosterone level was lost (Figure R5A). Also as expected, CRH immunostaining in the PVH failed to show any positive structure in the PVH in CRH-Cre:Ai9 reporter mice (tdTomato for CRH-Cre neurons and GFP for CRH, Figure R5B) . In CRH-Cre mice injected with Kir2.1 vectors to one side of the PVH, CRH immunostaining also failed to show any positive structure (Figure R5C). Consistent with CRH expression in terminals, CRH immunostaining showed strong positive structures in ME. In CHR-Cre::Ai9 mice, CRH expression exhibited even levels on both sides (Figure F5D). In contrast, in Kir2.1 mice, the side with more Kir2.1 expression exhibited less CRH expression (Figure F5E), suggesting that Kir2.1 inhibits CRH expression.

Figure R5



It is somewhat surprising that NachBac mice showed an exaggerated corticosterone levels during dark periods (Fig. R6A). However, as corticosterone represents a remote, as opposed to an immediate downstream indicator of CRH neuron activity, corticosterone levels may not exactly reflect CRH neuron activity levels, which is particularly the case for the HPA axis as corticosterone levels are known to be regulated by its own feedback inhibition at both CRH neuron and pituitary ATCH cell levels. Supporting this, previous studies on mice with PVH neuron specific deletion of glucocorticoid receptor (GR) (deletion of which is known to increase CRH expression and disrupt ACTH diurnal patterns) (Laryea *et al*, 2013), a condition similar to our mice with NachBac in terms of CRH expression levels (i.e. both will increase CRH expression), also causes an exaggerated corticosterone level during dark periods (Figure R7), a similar observation to ours shown here. We therefore directly examined CRH expression in the PVH as a proxy for neuron activity. As CRH is normally located at the terminals, immunostaining can't normally show CRH positive structures in the PVH (Figure R6C, top two rows). However, NachBac expression renders obvious CRH positive structures in the PVH under both day and night periods (Figure



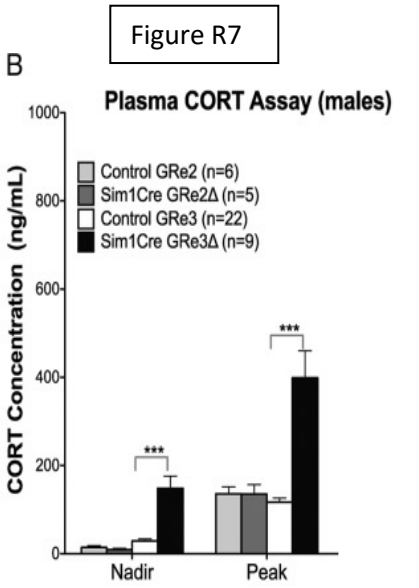


Fig. 3B from Laryea G et al., Mol Endocrinology, 2013. Black bars represent mice with GR deletion from PVH neurons.

R6C, bottom two rows, arrows), suggesting increased CRH expression by NachBac. We also compared CRH immunostaining in median eminence (ME) and found that mice with NachBac expression in the CRH exhibited more CRH immunostaining in ME (Figure R6B), again suggesting increased CRH expression by NachBac expression. These results provide additional support for the original data presented in Figs. 2 and 4 that 1) Kir2.1 expression reduces the activity and diurnal activity pattern of CRH neurons and 2) NachBac expression increases the activity and reduces diurnal activity pattern of CHR neurons.

5. If disrupted hypothalamic CRH neuron responsiveness contributes to DIO, why animals with their CRH neuron activity clamped at low or high levels did not show changes in their metabolic stage? It seems that the lack of CRH neurons responsiveness to a change in the nutritional environment is a consequence of DIO and does not present a causality component to obesity.

Response: We have to respectfully point out that both of our model with low (Kir2.1) and high (NachBac) showed changes in both feeding, energy expenditure and locomotion, compared to controls. In particular, NachBac mice showed obesity on chow diet, and while Kir2.1 mice didn't show obesity on chow diet, they were much more sensitive to DIO, compared to controls. As we discussed in the manuscript, the phenotype of Kir2.1 mice on chow diet may be counter-balanced by presumptive beneficial effects from lowering CRH neuron activity, as shown by their glucose phenotypes (Figure EV5), which is improved when on chow but dramatically reversed upon fed HFD.

Our fiber photometry data show that CRH neurons with Kir2.1 or NachBac expression exhibit largely diminished responsiveness (Figs. 2G-2J and Figs. 4G-4J) when measured at a time there is no body weight difference, arguing against a secondary effect to obesity. Both mouse models are more sensitive to DIO, suggesting a causal relationship to obesity development.

6. An experiment showing CRH PVH rescue of responsiveness should be performed in an attempt to link the lack of responsiveness to obesity.

Response: We appreciate the reviewer's point on the experiment of using rescuing approach to confirm the phenotype, which is also one of our research priorities. However, currently, we just don't have an effective means to achieve this goal. To do a rescuing experiment, we would have to generate a mouse model with increased amplitude in the diurnal pattern of CRH neuron activity, which would require a promoter that shows a diurnal activity pattern and a gene that encodes protein expression capable of altering neuron activity across 24 hrs. We are not aware of any reliable promoters/genes that can be used in combination for this purpose.

However, it is important to point out that, even without this rescuing experiment, the data presented in the current study are sufficient to draw the conclusion that lack of responsiveness contributes to DIO. We show that mice with CRH neuron chronically activated or inhibited both lead to DIO with reduced diurnal rhythms, ruling out a role for absolute activity levels of these neurons in DIO. Importantly, DIO reduces CRH neuron responsiveness, which is mimicked by both models.

Minor Comments

1. The entire manuscript should be carefully revised as it presents a high incidence of typos, divergent nomenclature viruses and citations standardized throughout the document.

Response: We have carefully read and made corrections on typos and nomenclatures.

2. CRH-Cre mouse is a well-established model, and the authors have to provide citations and background information.

Response: The information has been provided (Taniguchi *et al*, 2011).

3. Would be appropriate if the authors verify the co-localization of tdTom and CRH in the CRH-Cre::Ai9 reporter mouse.

Response: In this CRH-Cre line, Cre activity and CRH expression co-localization have been demonstrated previously (Wamsteeker Cusulin *et al*, 2013).

4. Chow and HFD ad lib animals should be demonstrated in Figure 1 alongside with fasted and refed mice.
Response: We have added c-Fos data in chow and HFD in new Figure EV1.

5. In Figure 2 there is some contradictory information. The authors first describe it as bilateral injection of Flex-EGFP-P2A-mNachBac and then claim that they used the contralateral side as control (second paragraph, page 5).

Response: This confusion has been clarified.

6. The Corticosterone basal level of the animals that were treated with DEX should be shown as the animals were adapted in the experimental chamber for 15 minutes prior to the recording, this change would be enough to elicitate stress response in the animals.

Response: We assume that the reviewer is referring to the experiments discussed in Figs. 1K-1M, Figs. 2I and 2J, and Figs. 4I and 4J. In these experiments we acclimated mice in behavioral chambers for 15 mins in order to reduce potential stress. Also, as our focus is on CRH neuron GCaMP signal and after 15 mins in the chamber, the GCaMP signal is stable before we inject DEX. Moreover, as we only focus on acute changes of CHR neurons to DEX (i.e. within mins), the baseline level of corticosterone, even different, will not confound data interpretation.

Referee #3:

1) There are a lot of moving parts to this study, and I think that the investigators are somewhere from showing that the artificial clamping of neuronal function in anyway mimics a HFD. The clamping of the neurons may simply have turned out to be a sophisticated way of 'knocking out' the function of the cells, therefore resulting in a phenotype. The response of the neurons to a high-fat diet is far more subtle. I understand why the authors have interpreted the data as such, but I think they should be more circumspect in their reporting.

Response: Our evidence on NachBac effects on CRH neurons strongly argue against the reviewer's suggestion on "knocking out" the cells. Our collective data on NachBac in 1) strong c-Fos expression, which is opposite to the Kir2.1 expression effect (Fig. 2D versus Fig. 4D); 2) more CRH expression, which is also opposite to the Kir2.1 expression effect (Figure R5 versus Figure F6 above); 3) Differential corticosterone profile (Figure R5 versus Figure F6 above); and 4) electrophysiological responses, which is

also opposite to the Kir2.1 expression effect (Figs. 2A-2C versus Figs. 4A-4C). These data collectively show that CRH neurons are either clamped at low levels (Kir2.1) or at high levels (NachBac), resulting in "knocking out" of their normal ability to respond dynamically to various stimuli, which causes susceptibility to DIO.

We agree with the reviewer on that the HFD feeding effect in disrupting neuron responsiveness is much weaker than NachBac or Kir2.1. We understand that the effect of genetic manipulations on CRH neuorns (NachBac and Kir2.1) is much greater than HFD. Also HFD feeding may also affect many other groups of neurons in the brain. Thus, we only claim that disrupted hypothalamic CRH neuron responsiveness contributes to diet-induced obesity.

2) Have the investigators measured what happens to CRH, either at the transcript of protein level, in response to all of the perturbations? HFD or the clamping? I think this would add weight to the argument that this could play a potential role in stress induced weight gain.

Response: As shown Figure R5 and Figure R6 in our response to Reviewer 2, CRH expression is reduced with Kir2.1 expression and increase with NachBac expression.

3) This is minor, but there are many typos and grammatical errors sprinkled throughout. This manuscript will need careful copyediting.

Response: We have carefully read and proof-edited the manuscript.

References:

Laryea G, Schutz G, Muglia LJ (2013) Disrupting hypothalamic glucocorticoid receptors causes HPA axis hyperactivity and excess adiposity. *Mol Endocrinol* 27: 1655-1665

Taniguchi H, He M, Wu P, Kim S, Paik R, Sugino K, Kvitsiani D, Fu Y, Lu J, Lin Y *et al* (2011) A resource of Cre driver lines for genetic targeting of GABAergic neurons in cerebral cortex. *Neuron* 71: 995-1013

Wamsteeker Cusulin JI, Fuzesi T, Watts AG, Bains JS (2013) Characterization of corticotropin-releasing hormone neurons in the paraventricular nucleus of the hypothalamus of Crh-IRES-Cre mutant mice. *PLoS One* 8: e64943

2nd Editorial Decision 17 April 2020

Thank you for the submission of your revised manuscript. We have now received the comments from all referees and I am pleased to tell you that all support the publication of your revised study. Only a few more minor changes are required before we can proceed with the official acceptance.

Zhiying Jiang is missing from the author contributions. NJJ is mentioned in the Acknowledgements but there is no matching name in the author list. Please correct/add.

There are two callouts for Fig EV 4N, which does not exist.

The figures are not of production quality. Please submit high resolution figures with your final manuscript file. For more information on figure preparation please see our guide to authors online: https://www.embopress.org/page/journal/14693178/authorguide#figureformat You also find a link there to a figure guide pdf file.

The legend for panel J of Fig EV3 is missing.

Please upload the source data as one file per figure.

Please remove DATA NOT SHOWN on page 5, as per journal policy.

Until the end of this month the EMBO reports reference style is still numbered. If you submit the final manuscript within the next week, please change the reference style to the numbered EMBO reports style. IF you need more time, you can use the new reference style. Links to both styles can be found in our guide to authors.

The funding info R21NS108091 is not in the manuscript file, please add it to the Acknowledgements.

I attach to this email a related ms file with comments by our data editors. Please address all comments in the final manuscript file.

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

Disrupted Hypothalamic stress Neuron Responsiveness Contributes to Diet-induced Obesity

The current obesity epidemic mainly results from high-fat high-caloric diet (HFD) feeding and may also be contributed to by chronic stress; however, the neural basis underlying stress-related diet-induced obesity remains unknown. Corticotropin releasing hormone (CRH) neurons in the paraventricular hypothalamus (PVH), a known body weight-regulating region, represent one key group of stress-responsive neurons. Here we show that HFD feeding blunts PVH CRH neuron responses to nutritional challenges as well as stress stimuli and dexamathesone, which normally produce rapid activation and inhibition of these neurons, respectively. We generated mouse models with the activity of these neurons clamped at high or low levels, both of which show HFD-mimicking, blunted PVH CRH neuron responsiveness. Strikingly, both models develop rapid HFD-induced obesity, associated with HFD-mimicking, reduced diurnal rythmicity in feeding and energy expenditure. Thus, blunted responsiveness of PVH CRH neurons, but not their absolute activity levels, underlies HFD-induced obesity, and may contribute to stress-induced obesity.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

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

REFEREE REPORTS

Referee #1:
I do not have additional comments. I apologise for the lateness of my review.

Referee #2:
All my concerns were appropriately addressed and I recommend the publication of this manuscript.

Referee #3:
The authors have responded satisfactorily to my concerns.

2nd Revision - authors' response 19 April 2020

The authors performed all minor editorial changes.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Qingchun Tong, PhD
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-49210V3

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 iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We chose sample size based on combination of literature reports and pilot experiments on the differnce between groups.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Yes.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We performed posthoc analysis and excluded those animals with off-target injections.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All animals used were from the same breeding pairs and randomly distributed between experimental groups. For body weight studies, we controlled for body weight to be comparable beteen groups before the onset of experiments.
For animal studies, include a statement about randomization even if no randomization was used.	Yes.
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.	All mouse study subjects were littermates and the body weight was comparable between groups before the onset of experiments. During body weight measurement, the animal ID was blinded to the experimentor.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	Yes.

USEFUL LINKS FOR COMPLETING THIS FORM

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

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

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

http://datadryad.org

http://figshare.com

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

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

http://biomodels.net/

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

Is the variance similar between the groups that are being statistically compared?	Yes.

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).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Included.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Included.
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.	Yes.

E- Human Subjects

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

F- Data Accessibility

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

G- Dual use research of concern

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