

Manuscript EMBO-2015-91942

Distinct preoptic-BST nuclei dissociate paternal and infanticidal behavior in mice

Yousuke Tsuneoka, Kenichi Tokita, Chihiro Yoshihara, Taiju Amano, Gianluca Esposito, Arthur J. Huang, Lily M.Y. Yu, Yuri Odaka, Kazutaka Shinozuka, Thomas J. McHugh, and Kumi O. Kuroda

Corresponding author: Kumi Kuroda, RIKEN Brain Science Institute

Review timeline:

Submission date:	07 May 2015
Editorial Decision:	11 July 2015
Revision received:	05 August 2015
Editorial Decision:	07 August 2015
Accepted:	07 August 2015

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.)

Editor: Karin Dumstrei

1st Editorial Decision

11 July 2015

Thank you for submitting your manuscript to The EMBO Journal. I am terribly sorry about the delay in getting back to you with a decision, but in this case it unfortunately took a bit longer than anticipated to get the referee reports back.

I have now received the referee reports on your manuscript and as you can see below both referees find the analysis interesting, well done and support publication here. They raise different issues that should be resolved, but none of them will take too much work to sort out. I would therefore like to invite you to submit a suitably revised manuscript.

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:
http://emboj.embopress.org/about#Transparent_Process

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

REFeree REPORTS:

Referee #1:

The study by Tsuneoka et al examines the role of various nuclei in the hypothalamus and related structures in parental behaviors. Using elegant neuroanatomy and c-Fos labeling, they find a striking correlation (and subsequent predictive power) in the pattern of c-Fos activation during parental care, parental neglect (infanticide), mating, and aggression. Based on the c-Fos studies, they focus their attention on the rhBNST and MPOA and their role in infanticide and parental care, respectively. Excitotoxic ablation of neurons in these regions appear to confirm observations with c-Fos. Optogenetic or pharmacogenetic activation of MPOA neurons appears to decrease infanticide, albeit at over a surprisingly long timeframe of several days. These are interesting findings and I am supportive of publication, but please see my comments below.

1) Both manipulations (ablation and activation) are done in a non-genetically targeted fashion. This is understandable because these represent early attempts to understanding the underlying neurobiology. However, these non-genetically targeted studies are always associated with many limitations, none of which are discussed in the manuscript. These limitations need to be acknowledged explicitly. These include bleed-over of neuronal loss in adjacent regions (very obvious in figures 3, 4) and similarly, stimulation of neurons outside the MPOA; the non-restricted nature of labeling is also evident in the fluorogold labeling.

2) The change in latency to infanticide is significant upon ablation of MPOA or rhBNST (figures 3F and 4F). However, the distribution of individual animals appears bi-modal, especially in 3F where it seems that the correlation may be skewed by outliers. This caveat needs to be acknowledged explicitly in the text or the authors should increase sample size.

3) It is unclear from the Methods how figure 7C was generated: what assays and outcomes were included to generate this matrix?

4) The attribution of the ventromedial hypothalamus as an aggressive center is cited as Lin et al 2011. However no markers were employed in this study to show that the relevant neurons were located in this region. The studies that identified the specific VMH neurons and aggression are Yang...Shah, Cell 2013 and Lee....Anderson, Nature 2014.

Referee #2:

The authors have submitted a very interesting and extensive study on the switch of infanticide to paternal behavior in male mice. They show that activation of the rhomboid nucleus of the BNST induces infanticide while activation of the cMPOA is important during paternal behavior. The authors applied a huge variety of modern techniques to address the above mentioned question. However, at some points, more elaborate descriptions of the methods or the behavioral circumstances are required.

Title:

Please add "in mice" to the title to point to the species used in the experiment

Abstract:

Line 13f.: The statement that optogenetic or pharmacogenetic activation of cMPOA suppressed infanticide in virgins is too strong. The authors hypothesized this suppression but could not prove it completely in the end. Therefore, the statement needs to be weakened.

Introduction:

General: Introductory words on the BNST are completely missing. Only the MPOA is covered.

Line 6: please change "parental" to "paternal" as female mice do show spontaneous maternal behavior.

Line 20: please add the precise name of the strain of the mice to the species.

Results:

General: please always indicate when speaking of virgin mice that you had male virgin mice.

Page 3, line 33: please add the line J or N to the C57BL/6 mice (also in the rest of the manuscript)

Page 4, paragraph 1: Please describe more clearly why you chose to identify the BNST nuclei using the various neurotransmitter systems. It is not clear why these neurotransmitters are specific to the

distinct nuclei. Additionally, the description of the transverse nucleus is lacking a neurotransmitter system similar to the other described nuclei.

Page 4, line 26: Please add "of" to the sentence.

Page 4, line 32: It is not completely clear from the structure of this part/paragraph that the following information is the description of the "pre-test". Please clarify accordingly.

Page 5, line 11: The authors need to indicate which triple staining was performed.

Page 7, paragraph 2: The approach of determining the behavior by neuronal activation patterns is indeed innovative and interesting. However, the reason to do so and the benefit are not well elaborated and need to be clarified.

Page 9, line 1: Please state which mRNA was measured by ISH.

Discussion:

General: in the results section, the latency to infanticide was multiple times affected by treatment. However, I am missing some discussion about potential meanings. Furthermore, any discussion about the non-parental stimuli/groups (mating, intermale aggression) is missing.

Page 12, line 12: I doubt that the male mice "choose" the behavior; please reword this part.

Page 12, line 31ff: The suggestion that these mice would serve as a new model seems to be very far-fetched and is not elaborated. Please remove or elaborate.

Page 13, paragraph 2: The whole paragraph seems to be a bit irrelevant in lights of the results. Furthermore, the authors point to the heterogeneity of the BNST and then assign one behavior to one nucleus. This does not fit to the heterogeneity and is misleading to believe that a certain nucleus is responsible for a certain behavior. Typically, the distinct nuclei of the BNST encode for a variety of behaviors and should not be limited to one behavior.

Material & Methods:

Page 17, line 17: Please indicate the units of the coordinates.

Page 17, line 18: Please state why the authors used two different infusion volumes.

Page 17, general: Please give more information on the stereotaxic surgery. Was the surgery performed under sterile conditions? Did the mice get antibiotics afterwards? How was the wound closed?

Page 18, line 20: Please check for orthography.

Page 19, lines 12-17: Did the authors use sense probes as negative controls? How did the authors control for specificity?

Page 22, general: Please indicate how the mice were housed after surgery.

Page 23, line 16ff: Please mention the name of the method (DREADD) also here (not only in results part)

Page 23, line 29: The authors need to give a reference for the drug. Furthermore, it is not clear if the drug can cross the blood brain barrier. Are 30 min sufficient to elicit effects within the brain?

Page 24, line 11: The use of Welch's t-test as test for post hoc comparisons seems inappropriate as it does not control for multiple groups. The use of standard post hoc tests like Bonferroni post hoc test seems more appropriate.

Figures:

General: It would be useful to indicate the distance from Bregma as well as landmarks like the anterior commissure or the lateral/3rd ventricle on histology slices whenever applicable.

Furthermore, a table with abbreviations would be helpful.

Legend to figures 3, 4: part "C" is not divided in top, middle and bottom. Please check.

Referees' comments and our point-by-point responses

Referee #1:

The study by Tsuneoka et al examines the role of various nuclei in the hypothalamus and related structures in parental behaviours. Using elegant neuroanatomy and c-Fos labelling, they find a striking correlation (and subsequent predictive power) in the pattern of c-Fos activation during parental care, parental neglect (infanticide), mating, and aggression. Based on the c-Fos studies, they focus their attention on the rhBNST and MPOA and their role in infanticide and parental care,

respectively. Excitotoxic ablation of neurons in these regions appear to confirm observations with c-Fos. Optogenetic or pharmacogenetic activation of MPOA neurons appears to decrease infanticide, albeit at over a surprisingly long timeframe of several days. These are interesting findings and I am supportive of publication, but please see my comments below.

Response: We appreciate the reviewer's positive assessment of our paper. The changes made to the main text according to suggestions from this reviewer are marked by red colour in the revised manuscript.

1) Both manipulations (ablation and activation) are done in a non-genetically targeted fashion. This is understandable because these represent early attempts to understanding the underlying neurobiology. However, these non-genetically targeted studies are always associated with many limitations, none of which are discussed in the manuscript. These limitations need to be acknowledged explicitly. These include bleed-over of neuronal loss in adjacent regions (very obvious in figures 3, 4) and similarly, stimulation of neurons outside the MPOA; the non-restricted nature of labelling is also evident in the fluorogold labelling.

Response: We have added the following sentences to the manuscript " Wu et all's study described direct manipulation of galanin-expressing neurons, perhaps explaining the more rapid and drastic effects of optogenetic stimulation they report." to the Discussion (page 12, lines 15-17). For the fluorogold labelling, the deposition sites of the fluorogold solution have been labelled on Figure 6A and 6F. In the present study, injection was made using only a small volume of fluorogold solution which makes it very difficult to discriminate the deposition site from the surrounding area that contains retrogradely labelled neurons.

2) The change in latency to infanticide is significant upon ablation of MPOA or rhBNST (figures 3F and 4F). However, the distribution of individual animals appears bi-modal, especially in 3F where it seems that the correlation may be skewed by outliers. This caveat needs to be acknowledged explicitly in the text or the authors should increase sample size.

Response: We have added the caveat to the legend of Figure 3, as follows "Please note that the distribution of each variable, in particular the latency of infanticide, shows a bimodal nature, and may skew the correlation index in the scattered plots." (page 28, lines 27-29).

3) It is unclear from the Methods how figure 7C was generated: what assays and outcomes were included to generate this matrix?

Response: We have added a sentence to the Result section, as follows "Figure 7C summarizes the results for each animal, and each square represents the behaviour during each test session, colour coded for infanticide (red), aggressive contact (yellow), ignoring the pup (light blue), and parenting (dark blue)." (page 9, lines 28-30).

4) The attribution of the ventromedial hypothalamus as an aggressive centre is cited as Lin et al 2011. However no markers were employed in this study to show that the relevant neurons were located in this region. The studies that identified the specific VMH neurons and aggression are Yang....Shah, Cell 2013 and Lee....Anderson, Nature 2014.

Response: We have replaced the references accordingly.

Referee #2:

The authors have submitted a very interesting and extensive study on the switch of infanticide to paternal behaviour in male mice. They show that activation of the rhomboid nucleus of the BNST induces infanticide while activation of the cMPOA is important during paternal behaviour.

The authors applied a huge variety of modern techniques to address the above mentioned question. However, at some points, more elaborate descriptions of the methods or the behavioural circumstances are required.

Response: We sincerely thank the reviewer for the kind words. The changes made to the main text according to this reviewer are marked by blue colour in the revised manuscript.

Title:

Please add "in mice" to the title to point to the species used in the experiment

Response: We have added "in mice" to the end of the title. (page 1, line 3).

Abstract:

Line 13f: The statement that optogenetic or pharmacogenetic activation of cMPOA suppressed infanticide in virgins is too strong. The authors hypothesized this suppression but could not prove it completely in the end. Therefore, the statement needs to be weakened.

Response: We have changed the sentence to "Optogenetic or pharmacogenetic activation of cMPOA attenuated infanticide in virgin males." (page 2, lines 13-14).

Introduction:

General: Introductory words on the BNST are completely missing. Only the MPOA is covered.

Line 6: please change "parental" to "paternal" as female mice do show spontaneous maternal behaviour.

Line 20: please add the precise name of the strain of the mice to the species.

Response: We have added the requested information accordingly.

Results:

General: please always indicate when speaking of virgin mice that you had male virgin mice.

Response: The text was revised accordingly.

Page 3, line 33: please add the line J or N to the C57BL/6 mice (also in the rest of the manuscript)

Response: We added J to the C57BL/6 throughout the manuscript.

Page 4, paragraph 1: Please describe more clearly why you chose to identify the BNST nuclei using the various neurotransmitter systems. It is not clear why these neurotransmitters are specific to the distinct nuclei. Additionally, the description of the transverse nucleus is lacking a neurotransmitter system similar to the other described nuclei.

Response: The neurochemical markers used to identify the BST subnuclei were chosen according to the previous literature describing the rat BST (Dong et al, 2001; Ju & Swanson, 1989; Ju et al, 1989) (page 4, lines 2-3). As this reviewer correctly pointed out, a given neurotransmitter is not necessarily specific to each BST subnucleus, nor each BST subnucleus may not be defined solely by the neurochemical features; for example, the transverse nucleus of the BST was defined using its cyto-architectonic character as "The transverse nucleus of BST (BSTtr) was located ventral to the BSTrh, and identified by Nissl staining as a cluster of neurons that were oriented transversely". (page 4, lines 10-12).

Page 4, line 26: Please add "of" to the sentence.

Response: Done accordingly. (page 4, line 26)

Page 4, line 32: It is not completely clear from the structure of this part/paragraph that the following information is the description of the "pre-test". Please clarify accordingly.

Response: For clarification, we have modified this part as follows; "To examine c-Fos expression induced by pup exposure in virgin males and fathers, these mice were first pre-tested to determine their pup-directed behaviour. All fathers exhibited paternal behaviour on postpartum day 3. The majority of virgin males committed infanticide, while a small fraction (6.5%) of virgins spontaneously performed paternal behaviour in the pre-test, as expected from our previous study (Tachikawa et al, 2013). Clearly infanticidal virgin males (n = 7), paternal virgin males (n = 6) and fathers (n = 11) were selected and were singly housed for two days to allow the c-Fos expression induced by the pre-test to return to baseline levels (Morgan et al, 1987) (Numan & Numan, 1994), and then they were exposed to three donor pups for two hours. The pup-directed behaviour during this main experiment was essentially consistent with the pre-test as shown previously (Tachikawa et al, 2013)." (page 4, line 32 - page 5, line 9).

Page 5, line 11: The authors need to indicate which triple staining was performed.

Response: The sentence was revised as "the brains were subjected to triple immunohistochemical labelling (Fig 2A–Y, Fig S1) for c-Fos, VIP and galanin, to quantify the c-Fos positive neurons in each nucleus". (page 5, lines 15-17).

Page 7, paragraph 2: The approach of determining the behaviour by neuronal activation patterns is indeed innovative and interesting. However, the reason to do so and the benefit are not well elaborated and need to be clarified.

Response: The underlined part was added to the relevant sentence; "We next tested whether the males' recent social behaviour could be deduced simply by measuring the neuronal activation pattern of the preoptic-BST nuclei, this kind of analysis lays the groundwork for possible applications to detect or prevent infanticide by neuroimaging in the future." (page 7, lines 21-23).

Page 9, line 1: Please state which mRNA was measured by ISH.

Response: The following words were added to the sentence; "by double fluorescent in situ hybridization (ISH) for GAD67 and VGLUT2 mRNAs". (page 9, lines 11-12).

Discussion:

General: in the results section, the latency to infanticide was multiple times affected by treatment. However, I am missing some discussion about potential meanings. Furthermore, any discussion about the non-parental stimuli/groups (mating, intermale aggression) is missing.

Response: For the former point, we have added "The present study revealed that the BSTRh was specifically activated by infanticide, as well as that BSTRh bilateral lesions led to a significant increase in infanticide latency." (page 14, lines 3-5)

For the latter point, we have added the underlined part to the first sentence of the last paragraph of the Discussion, "In this study we demonstrated the remarkable capacity of the activation pattern in the four preoptic-BST nuclei to retroactively detect major repertoires of social behaviours of male mice." (page 14, lines 27-29)

Page 12, line 12: I doubt that the male mice "choose" the behaviour; please reword this part.

Response: The word "choose" was replaced to "express only one at a time". (page 11, line 25)

Page 12, line 31ff: The suggestion that these mice would serve as a new model seems to be very far-fetched and is not elaborated. Please remove or elaborate.

Response: The sentence "The behavioural transition from infanticide to parenting in male mice would serve as a new model system for the selection processes of mutually exclusive behaviours based on social memory in general" has been removed.

Page 13, paragraph 2: The whole paragraph seems to be a bit irrelevant in lights of the results. Furthermore, the authors point to the heterogeneity of the BNST and then assign one behaviour to one nucleus. This does not fit to the heterogeneity and is misleading to believe that a certain nucleus is responsible for a certain behaviour. Typically, the distinct nuclei of the BNST encode for a variety of behaviours and should not be limited to one behaviour.

Response: We appreciate this thoughtful comment. This paragraph was cut short and the relevant sentence has been modified as follows;

(Original) "Our data reveal that each BST nucleus has a clear association with a specific social behaviour, BSTpr/mating (and parenting), BSTrh/infanticide, BSTal and BSTtr/aggression and mating, and BSTov/no clear correlation with the social behaviours examined in this study."

(Revised) "The present study revealed that the BSTrh was specifically activated by infanticide, as well as that BSTrh bilateral lesions led to a significant increase in infanticide latency." (page 14, lines 3-5)

Material & Methods:

Page 17, line 17: Please indicate the units of the coordinates.

Response: "mm" was added to each coordinate throughout the text.

Page 17, line 18: Please state why the authors used two different infusion volumes.

Response: This was simply because the size of the cMPOA and BSTrh were different. For clarification, we have modified this sentence to "The injection volume was 15 nl for cMPOA and 20 nl for BSTrh." (page 18, lines 6-7)

Page 17, general: Please give more information on the stereotaxic surgery. Was the surgery performed under sterile conditions? Did the mice get antibiotics afterwards? How was the wound closed?

Response: The surgery was performed under sterile condition and without antibiotics. The wound was closed by silk suture. This information and some details were added to this paragraph and coloured in blue. (page 18, lines 7-8)

Page 18, line 20: Please check for orthography.

Response: Thank you very much for careful reading. This sentence was corrected to 3,3' – diaminobenzidine (DAB). (page 19, line 7)

Page 19, lines 12-17: Did the authors use sense probes as negative controls? How did the authors control for specificity?

Response: The specificity of each ISH probe was confirmed by comparison with previous literature and with the Allen Mouse Brain Atlas database, rather than testing each sense probe. This information was added to the Materials & Methods section (page 19, lines 27-29).

Page 22, general: Please indicate how the mice were housed after surgery.

Response: The underlined part was added; "Subject virgin male mice were group-housed until 3 months of age at the start of experiment, and then were single-housed 2 days before the preparatory pup exposure assay (pre-test) and throughout the experiment." (page 24, lines 25-27).

Page 23, line 16ff: Please mention the name of the method (DREADD) also here (not only in results part)

Response: Done accordingly. (page 24, lines 23-24).

Page 23, line 29: The authors need to give a reference for the drug. Furthermore, it is not clear if the drug can cross the blood brain barrier. Are 30 min sufficient to elicit effects within the brain?

Response: A reference for CNO (Armbruster 2007) and the previous paper describing the pharmacodynamics of CNO in the mice in vivo (Alexander 2009) were added. The latter described that the peripherally-injected CNO could cross the blood-brain barrier and exert an effect on the behaviour within 30 min.

Page 24, line 11: The use of Welch's t-test as test for post hoc comparisons seems inappropriate as it does not control for multiple groups. The use of standard post hoc tests like Bonferroni post hoc test seems more appropriate.

Response: Actually we controlled for the comparison of multiple groups using the Holm's sequential Bonferroni method. For clarification, we have added the term "sequential Bonferroni" (page 25, line 23).

Figures:

General: It would be useful to indicate the distance from Bregma as well as landmarks like the anterior commissure or the lateral/3rd ventricle on histology slices whenever applicable. Furthermore, a table with abbreviations would be helpful.

Response: The distance from Bregma has been added to the legends of Figure 1, 2, S1, and the anterior commissure / fornix/ 3rd ventricle has been marked in Figure 4A, 6B, 6G, 7G, S1, S3, S4. A table for listing abbreviations was added (Table I).

Legend to figures 3, 4: part "C" is not divided in top, middle and bottom. Please check.

Response: We are sorry for the mistake. The beginnings of the legends have been corrected from "A-C" to "A-B".

Other changes

*We have added several methodological details and sample size indication to the text (marked as **GREEN** colour), according to the "EMBO Author Checklist".*

In addition, some changes are made for the current address for the authors.