Wednesday, July 4, 2018

JoVE

Re: Assessing Cellular Stress and Inflammation in Discrete Oxytocin-Secreting Nuclei in the Neonatal Rat Before and After First Colostrum Feeding

Dear Vineeta Bajaj,  
  
Thanks to you and the reviewer for the careful review of our manuscript. Below we detail our responses to reviewer feedback. We have made the suggested revisions and believe this paper has benefited greatly from the review process.

Thank you for your consideration of our paper.

Respectfully,

Benjamin Y. Klein, MD and Martha G. Welch, MD

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**Editorial comments:**  
Changes to be made by the Author(s):  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript.

2. Please revise lines 93-95, 221-227, 237-239, 400-401, and 410-412 to avoid previously published text.

We have revised the text for lines 93-95 , 400-401, and 410-412. The text for lines 221-227 and 237-239 is methods text and remains as is.

3. Figures: Please list the panels in order from left to right and then top to bottom. Please be consistent throughout.

We have made this edit, which includes an updated Figure 4.

4. Figure 2: Please line up panels B and C. Please define error bars in the figure legend.

This figure has been updated and the legend updated to define error bars.

5. Figure 3: Please define error bars in the figure legend.

The figure legend has been updated.

6. Figure S1: Please change “hr” to “h” and include a space between the number and time unit (i.e., 1 h, 2 h, etc.).

We have made this edit.

7. Please revise the Introduction to include all of the following:  
a) A clear statement of the overall goal of this method

We have added a statement (Lines 90-92).

b) The rationale behind the development and/or use of this technique

We have added this (Lines 92-96).

c) The advantages over alternative techniques with applicable references to previous studies

We have added this text (Lines 96-100)

d) A description of the context of the technique in the wider body of literature

We have added this (Lines 100 – 112).

e) Information to help readers to determine whether the method is appropriate for their application

We have added this (Lines 114-123)

8. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.  
done

9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Done

10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done.

11. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.  
For example: Bio Rad, ProteinSimple, WES-Mouse 12-230 master kit, Cell Signaling Technology (CST), Inc., Millipore, BIORBYT, Charles River Laboratories, etc.

Done

12. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Done

13. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Done

14. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have added additional detail.

15. Lines 135 and 151: Please move material information to the Table of Equipment and Materials.

Moved.

16. 1.1.2/1.1.3/1.3.2: Please break up into sub-steps and add more details to each sub-step.

We have added additional substeps.

17. 1.2.2: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Done

18. 1.3.1: Please remove the weblink which contains commercial language. Please add more details to this step.

Done

19. Lines 229-249: Unclear what we can show here. Please describe the actions.

We have added additional detail.

20. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

Done (Lines 295-298)

21. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique

Done

22. JoVE article does not have a Conclusion section. Please move information in the Conclusion section or Results or Discussion section.

done

23. References: Please do not abbreviate journal titles.

done  
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
To study the effect of colostrum oxytocin in gut on the activity of oxytocin receptor-rich brain regions, authors used a novel method for punching neonatal brain nuclei and then assessed BiP, eIF2a, NF-kB and IkB expressions using Western blotting before and after first colostrum feeding. The results showed that the stress modulating mechanisms previously observed in newborn gut enterocytes appear to be mirrored in some OTR-rich brain regions. Under nutrient deficiency the STN and PVN may utilize a different phosphorylation mechanism from other regions and be refractory to the impact of nutrient insufficiency. The authors concluded that brain responses to nutrient insufficiency stress are offset by signaling from colostrum-primed enterocytes. This work does show some innovation in the methods of sampling and in-capillary protein assay and is useful for establishing a theory that nutrient-insufficiency-related metabolic stress is a body-wide stressor. However, serious concerns about the methods and analyses should be addressed.  
  
Major Concerns:  
1. Feeding itself, even without milk or oxytocin, can strongly influence vagal input and brain activity (see Line 92). Thus a milk control containing no oxytocin or liquid without nutrients should be given.

We are grateful to the author for raising this important point. We did not pursue a “sham-feed” for two reasons. Frist, at Day 0 – 1 the vagal inputs is not yet developed fully and, second, the handling required for a sham-feed and equivalent handling in the primed group would also impact our measures and data interpretation.

2. Line 164, the sampling time between the unprimed and primed pups differed by 3-5 hours, which casts the doubt that the priming effect could reflect the natural developing process or adaptation process to the external environment. Thus, it is necessary to set an unprimed control group wherein sampling time is the same as the primed group. Alternatively, the authors may consider to provide the priming at the same time of the unprimed group.

We agree with the authors that the timing between primed and unprimed groups requires further evaluation. We chose to sacrifice unprimed pups prior to any stress response to eliminate the impact of stress related to the pup’s request for the first feed on gene expression. We will explore this feature in a subsequent paper.

3. Line 172: why was "artificial cerebrospinal fluid with 203.3mM MgCl 2 -6H2O used? No dehydration occurred?

Incubation in ACSF is necessary as a baseline for future options to examine the signaling response to selected inhibitors or stimulants for any signaling pathway.

4. In identification of said brain regions, histological presentation is necessary since the ordination or the surface marker of the skull was not showed.

We have indicated the distance from bregma, which allows for the identification.

5. Line 192, the meaning of "this extraction cocktail should contain protease inhibitors and phosphatase inhibitors" is not clear. Did it "contained" or did not contain" the inhibitors?

We have corrected, it contained inhibitors.

6. Line 109-201: "Protein Simple kit for Biotin-labeling of the total proteins concomitantly during electrophoresis to measure protein concentration, as opposed to adjusting the protein concentration to equal volumes in advance" raises the question if the quantification is valid.  
As shown in Figure 2 that changes in the areas of eIF2a and p-eIF2a are almost the same, and so does the total protein (SOPT and MPO are exceptional). Without a visual hint of clear changes, how could the authors get the values shown in the bar graphs? Are the exemplary bands representative? If the WES detected the density correctly, a loading control with adjusting the protein solution to equal concentration/amount should be presented, at least.

The tissue yield of some brain nuclei is very small to normalize against protein.

7. Discussion should address the comments listed above.

We have had to substantially modify the discussion to meet journal style and discuss primarily the technique. We have added some of this detail to the introduction.  
  
Minor Concerns:  
Line 177: the name of brain atlas referred should be given.

We have added.  
  
  
**Reviewer #2:**  
Manuscript Summary:  
This is modification of current techniques used to determine protein levels in brain tissue.  
  
Major Concerns:  
While present in the introduction, there is no premise/rationale presented in the abstract. There is nothing to pull the reader in to the topic of the research.

We have made these additions. Thank you for bringing this to our attention.  
  
Minor Concerns:  
1. The grammar is generally good, but could use an overall revision for a few minor issues.

We have given the manuscript a proofread.

2. Line 169, is this with a standard razor blade?

Yes, we have corrected.

3. Lines 203-206. This sentence is difficult to comprehend.  
This sentence has been removed in accordance with journal requirements to remove any commercial information.

4. Line 208, should this be -80C?

Yes, thank you for noticing, this has been corrected.

5. The graphs with asterisks are visually challenging. I'm not sure if the use of letters would be preferable by all, but alternative designs should be explored.

We have improved.  
  
  
**Reviewer #3:**  
Manuscript Summary:  
Traditionally Oxytocin was known only to involve in milk production and delivery. But the ever increasing roles of Oxytocin is further supported by the present study.  
Although there are other techniques such as single cell or brain slice approaches currently popular, but the authors put forward a detailed description of an efficient method to specifically isolate different OTR-rich regions within the rat-brain. Expression of different signaling proteins were tested in different brain regions and were compared to their already published data from gut. Based on their results, authors concluded that cell signaling in the brain associated with nutrient and hormonal insufficiency during the first hours following birth is similar to the gut under similar conditions.  
The method is well described and is probably reproducible, as authors claim. This method can probably be used to develop new protocols in different tissues too.  
  
All the best  
  
Thanks  
  
Major Concerns:  
NONE  
  
Minor Concerns:  
NONE

1 Hansson, J. *et al.* Time-resolved quantitative proteome analysis of in vivo intestinal development. *Mol Cell Proteomics.* **10** (3), M110 005231, doi:10.1074/mcp.M110.005231, (2011).

2 Mochizuki, K., Yorita, S. & Goda, T. Gene expression changes in the jejunum of rats during the transient suckling-weaning period. *J Nutr Sci Vitaminol (Tokyo).* **55** (2), 139-148 (2009).

3 Rinaman, L., Banihashemi, L. & Koehnle, T. J. Early life experience shapes the functional organization of stress-responsive visceral circuits. *Physiol Behav.* **104** (4), 632-640, doi:10.1016/j.physbeh.2011.04.008, (2011).

4 Dobbing, J. Boyd Orr memorial lecture. Early nutrition and later achievement. *Proc Nutr Soc.* **49** (2), 103-118 (1990).

5 Rinaman, L. Hindbrain noradrenergic A2 neurons: diverse roles in autonomic, endocrine, cognitive, and behavioral functions. *Am J Physiol Regul Integr Comp Physiol.* **300** (2), R222-235, doi:10.1152/ajpregu.00556.2010, (2011).

6 Walker, C. D., Toufexis, D. J. & Burlet, A. Hypothalamic and limbic expression of CRF and vasopressin during lactation: implications for the control of ACTH secretion and stress hyporesponsiveness. *Prog Brain Res.* **133** 99-110 (2001).