Author’s response to reviews

Title: Influence of isoflurane exposure in pregnant rats on the learning and memory of offsprings

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Author’s response to reviews:

The authors’ response letter has been included as a supplementary file.

Dear Editors and Reviewers:

Thank you for your letter and for the reviewers’ comments concerning our manuscript entitled “Paper Title” (ID: BANE-D-17-00295). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction which we hope meet with approval. Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the reviewer’s comments are as flowing:

Responds to the reviewer’s comments:

Reviewer 1: Misha Perouansky, MD (Reviewer 1):

Introduction:

1. Tighten references, please quote only the most relevant one for the statement you would like to support e.g. #2 seems sufficient, #1 unnecessary. Please select the most appropriate and highest quality publication in the best journal e.g. refs 9/10 - both necessary? be especially critical when quoting papers written by the authors of this study e.g. We have deleted references 9 according to the Reviewer’s suggestion. (Introduction section, line 20, page 3)
2. Most lab studies in rodent are done 1 week postnatal - place this date into the human pregnancy timeframe with respect to brain development. Ref 11 is not a good source to confirm but ref 12 is. This should be in the introduction when you explain.

We have deleted references 11 and this section has been moved to introduction according to the Reviewer’s suggestion. (Introduction section, line 23, page 3)

Methods

1. What does 'predesigned' gas mean?

Considering the Reviewer’s suggestion, the “predesigned gas” has been amended to “predfilled gas (According to the following groups)”, it means : 50% O2 was administered in the control group; 1.3% isoflurane was administered in the Iso1 group (50% oxygen, balanced with nitrogen); 2.0% isoflurane was administered in the Iso2 group (50% oxygen, balanced with nitrogen). (Methods section, line 17, page 5)

2. What is the source for the claim that most women undergo surgery at 12-16 weeks?

The source is from this following references: “An RF, Fang J. Pregnancy laparoscopic surgery. Journal of International Obstetrics and Gynecology 2003; 30: 144-7.” This references is a Chinese references, maybe it couldn’t find from PubMed, so we did not cite this references at first. Thank you for your comments.

3. Rats used in the 'further study' cannot be also part of a pilot study. The whole point of the pilot is to find out what works and perhaps to estimate the number needed for statistics.

We are very sorry for our incorrect writing, and We have corrected that “Rats used in pilot study will not be used for formal study.” (Methods section, line 4, page 6)

4. How did the randomization work?

The pregnant rats were assigned completely random. Every pregnant rat’s male offsprings were equal assigned two groups (28d and 90d) completely random. Considering the Reviewer’s suggestion, it has been amended to “At 28 days after birth (P28), every pregnant rat’s male offsprings were equal assigned two groups completely random, one group were for Morris water maze test to evaluate memory and learning. Another group were housed for a total of 90 days (P90) and then received Morris water maze (MWM) test.” (Methods section, line 9, page 6)

5. What was the litter size and sex distribution in the three experimental groups? What were the numbers of healthy / unhealthy male rats?
The litter size and sex distribution in the three experimental groups is in the follow table, and all the male rats are healthy. Considering that this table is not the main data in this article, it is not shown. Thank you for your comments.

6. When you say 'resuscitate' you actually mean 'recover'.

We have amended “resuscitate” to “recover” according to the Reviewer’s suggestion. (Methods section, line16, page5 and line7, page6)

7. If you followed a published protocol for interpreting the different aspects of the MWM interpretation, please indicate what the statement e.g. 'The swimming distance in the platform quadrant reflects spatial localization and the times of crossing the platform reflects the accuracy of spatial memory' is based on.

For this question, we think that many references suggest that MWM is a common tool for evaluating memory, especially the swimming distance in the platform quadrant and the times of crossing the platform. “The swimming distance in the platform quadrant reflects spatial localization and the times of crossing the platform reflects the accuracy of spatial memory” is the conclusion that I have drawn from the results of the study. Thank you for your comments.

8. How many rats were excluded 2/2 'visual problems' (how do you know they were visual?) in the different groups?

There was 1 rat excluded with visual problems, because it was swimming always stick to pool wall when the platform was above the water surface. Thank you for your comments.

9. How were the rats housed during development? How many to a cage? Standard or enriched environment?

The offsprings with the mother housed in a cage before 21 days. After 21 days, 5 rats will be put in a standard environment cage. Thank you for your comments.

10. How can you exclude rats after randomization? Figure 1 indicates that pregnant rats were randomized. The description refers however to exclusions of animals 2/2 poor performance. Specifically, would inclusion of the 'poorly performing' rat in the control group have abolished any significant differences in behavioral experiments

The poorly performing rat was swimming always stick to pool wall when the platform was above the water surface. And I recounted the results, if the rat was not excluded, the results have not changed much. Thank you for your comments.

Results

1. What is Mirros water maze?
The results of Mirros Water Maze is in the follow table. The figures of the results are in Fig2 and Fig3 of the manuscript. (Figure section, page18,20)

2. Fig 2 legend 'what's the percentage of platform quadrant'

The percentage of platform quadrant is the swimming distance in the fourth quadrant / total swimming distance. Thank you for your comments.

3. Fig 2. There is no difference between control and ISO 1 and a striking difference between ISO1 and ISO2 for one parameter (platform crossing) at both time points (2b and 3b). It is notable that at the same time the complementary measure i.e. 2c/3c (I do not exactly what % target quadrant means) shows no difference between any measures. Is this expected based on the theory underlying these assays? Please provide a convincing neuropsychologically / learning theory-based interpretation for why these two measures diverge and how the two tests are related from the point of view of neural substrate? How can one yield such a different result than the other and be so consistent at P28 and P90?

Many references suggest that MWM is a common tool for evaluating memory, especially the swimming distance in the platform quadrant and the times of crossing the platform. And from the results of the study, I came to the conclusion: In the spatial navigation test, the proportion of swimming distance in the platform quadrant to the total swimming distance reflects the capability of spatial location, and the times of crossing the platform reflects the accuracy of spatial memory. Thank you for your comments.

4. The same 'outlier' is found in the pCREB assay. How does one interpret the lack of a 'dose response.'

CREB can only participate in the formation of memory after phosphorylation, and the increase of pCREB is associated with the times of crossing the platform of MWM. Thank you for your comments.

5. Could you provide the individual values for these experiments? With other words, does individual performance in the water maze correlate with pCREB levels across experimental groups? Does a poorly performing rat in the control group have lower or higher pCREB expression than a well-performing rat in ISO2? Please cite literature showing that poor performance in a learning/memory task correlates with CREB/pCREB ratios

In our study, we found a poorly performing rat in the have lower pCREB expression than a well-performing rat, Whatever which group it is. Considering the Reviewer’s suggestion, references 34 has been cite showing that poor or good performance in a learning/memory task correlates with pCREB. (Discussion section, line23, page12)

Discussion:
1. The Clancy study does NOT claim that whole brain development is equivalent at certain age between rodents and humans. Clancy et al. are very careful to state that different parts of the brain reach certain milestones at different ages in different species i.e. brain development does not proceed at a uniform, easily comparable pace.

Considering the Reviewer’s suggestion, we have increased the references 11. （References section, line 12, page 11）

2. In all animals, a substantial proportion of neurons undergo apoptosis during normal development. Please be more accurate.

Considering the Reviewer’s suggestion, this section has been amended. （Discussion section, line 3, page 11）

3. You repeat the 'lack of guideline' issue - what kind of studies would you envisage that could provide 'guidelines' for humans? Is this feasible? Shouldn't this be based on evidence of a potentially detrimental effect in humans? If you insist (and I think these repeated inferences do not strengthen this MS) on this analogy - how relevant is a multi-hour exposure to these doses of isoflurane for the type of procedures commonly performed in pregnant women?

For this question, we think there are possibilities that pregnant patients may end up in critical condition with prolonged intubation requiring prolonged periods of anesthesia sedation. Thank you for your comments.

4. Roughly speaking, your experiments compare 3 MAC hours of isoflurane to 4.5 MAC hours occurring weeks prior to your CREB-assay. The similarity of the behavioral results with the biochemical one (i.e. a quantitative qualitative difference between the two doses of isoflurane) while not impossible, are not easy to understand. Are you postulating a 'threshold' effect captured by fortuitous choice of these two doses which affects only one of the two learning assays?

For this question, we think that in clinical practice, we usually use Iso<1.5 MAC, but we should maintain Iso>1.0 MAC in anesthesia, so we select these two concentrations for comparison in this study. Thank you for your comments.

5. pCREB/CREB are certainly important biochemical correlates of learning and memory. However, a single isolated assay does not provide a waterproof link especially considering the lack of a dose-response relationship. How does, as you say, can an effect on CREB phosphorylation in utero be still visible and affect behavior weeks later after all the experiences the animals had after birth? Phosphorylation is a dynamic process with high turnover times. A deeper explanation of CREB physiology would be helpful.

Considering the Reviewer’s suggestion, this section has been amended. （Discussion section, line 12, page 12）
Special thanks to you for your good comments.

Rany Makaryus, MD (Reviewer 2):

Major concerns:

1) It is unclear in the methods whether all groups (Iso1, Iso2, and control) had a subgroup of rats who had arterial lines placed the day before anesthesia exposure. Furthermore, how many were used in each group? Were the catheters then subsequently removed; when and how? This can be partly resolved potentially by including this in figure 1, which defines the maternal groups and experimental design. Finally on this point, the results of the BPs / ABGs were not presented, but this would be very helpful to see to determine the quality of anesthesia delivery.

Thank you for your comments. In the pilot study, each group had 6 pregnant rats, all the pregnant rats in the pilot study were put to death after the data collection was completed, and they are not included in the formal study. In the formal study, there are 16 pregnant rats in each group. The results of the BPs / ABGs are in the follow table.

2) The discussion (pg 16) states, "However, in clinical practice, it is infeasible that pregnant women received anesthesia but did not receive surgery and that surgery is performed under anesthesia for several weeks." This statement is too broad and may be over-reaching. It can be better worded, as there are possibilities that pregnant patients may end up in critical condition with prolonged intubation requiring prolonged periods of anesthesia sedation. This also negates the point of performing this study. I think it can be argued that though there is not a clear correlation to clinical practice, this is an important finding that needs further investigation.

We have re-written this part according to the Reviewer’s suggestion. (Discussion section, line7, page14)

Minor concerns:

1) In the methods section (pg. 7) and discussion section (pg. 13), the use of the term "middle trimester" is unusual, might consider utilizing the term "second trimester"

Considering the Reviewer’s suggestion, we have amended “middle trimester” to “second trimester”. ( Introduction section, line2, page4 and Discussion section, line15,16, page11)

2) The use of the word "resuscitate" on pages 7 & 8 is not correct, I assume this is referring to the recovery from anesthesia - should be reworded.

Considering the Reviewer’s suggestion, we have amended “resuscitate” to “recover”. ( Methods section, line16, page5 and line7, page6)
3) Figure 1 is not referred to in the manuscript.

Considering the Reviewer’s suggestion, we have added “Figure 1”. (Methods section, line20, page7)

Special thanks to you for your good comments.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. And here we did not list the changes but marked in red in revised paper.

We appreciate for Editors and Reviewers’ warm work earnestly, and hope that the correction will meet with approval.

Once again, thank you very much for your comments and suggestions.

Best wishes,

Ping Zhao, MD