Author’s response to reviews

Title: An in vivo animal study assessing long-term changes in hypothalamic cytokines following perinatal exposure to a chemical mixture based on Arctic maternal body burden.

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Author’s response to reviews:

Please find attached a revised version of our manuscript, along with a detailed letter outlining point-by-point our responses to the reviewer comments.

Response to Reviewer 1

1. Abstract: the first sentence of Methods is the objective of the study, not methods. All sentences of the Abstract should be more concise (i.e. no statistical details, no rationale for cytokines etc).

Response: We included statistics in the abstract owing to journal regulations. From the instructions to authors for this journal “The Abstract of the manuscript should not exceed 350 words and must be structured into separate sections: Background, the context and purpose of the study; Methods, how the study was performed and statistical tests used....”

2. We are not at all clear on what is being referred to “..no rationale for cytokines..”, as this does not appear in the abstract.

3. The last sentence of the first paragraph – please, give more details about pesticides (type), are these citations all relevant for the present research question?

Response: The following has been added to the sentence to show that the current state of data is generally unable to specifically pinpoint specific agents in Parkinson’s disease: “...use of pesticides across a broad classes of insecticides (e.g rotonone), herbicides (e.g. paraquat) and rodenticides...”

4. Page 6, 1st paragraph, last sentence – what is the relevance of the information about multiple sclerosis?
Response: We believe this sentence to be of significance given that it mentions the few studies that show neurological disease was associated with living in Northern Canadian regions, which of course is our target population from which based our chemical mixture.

Methods:
5. Page 9, last paragraph – it would be interesting to know the nature of “plastic cages”, knowing the ability of plastics to leach some potentially toxic additives, i.e. BPA, even at room temperature.

Response: The issue of BPA from standard animal cages is a contentious one. There is some indication that the levels are very low are unlikely to be of relevance here. Moreover, there would need to be some interaction between the treatments administered in the current study and seepage of BPA for this to be a problem for the current study. Finally, the issue of BPA seepage from plastic cages is analogous to potential co-exposure to metals, organics, etc contained in standard laboratory bedding, water supplies, and food supplies. While this is an interesting research question, it is well beyond the scope of the current study, and indeed most toxicity studies not addressing this specifically, especially considering that current evidence indicates that this is not a significant issue for most non-endocrine endpoints and indeed many endocrine endpoints.

6. The rationale on the choice of hypothalamus should be moved to the Background.

Response: This has now been moved to the last paragraph of the background section on P.7

7. Page 10 – “As described…”, this and the following sentence should be removed from this section entitled “Chemical administration procedures” or another section should be created to describe the overall experimentation protocol.

Response: These sentences have now been removed.

8. “In separate groups of aged female rats…” – it is not clear if there was animals from the same experiment, which was described before or a different experimentation, it would be useful also to indicate the age of animals.

Response: The sentence has been modified to read: “On PND 208-212, a separate set of female litter-mates were challenged with the bacterial endotoxin, lipopolysaccharide (LPS; 48 ug/kg; i.p.) and 90 minutes later were sacrificed by decapitation and the hypothalamus was dissected…”.

9. Table 1 should be moved to Tables section.
Response: Table has been moved to the end of the manuscript to the section with the figures.

10. Statistical analyses: why Tukey’s test was selected for multiple comparisons? If all exposed groups are compared to the control, Dunnett’s test would be more appropriate.

Response: We have re-written the statistical methods section. As now explicitly mentioned, we are not simply interested in comparing all groups to the vehicle controls. Although this is an important comparison, we are also interested in assessing whether the PCB + MeHg group differs from those that received the PCBs or MeHg alone, or the full mixture differs from the remaining groups.

“All data were analyzed using ANOVA followed by Tukey’s post hoc comparisons where appropriate. In effect, we assessed whether 1. perinatal treatment with PCBs or MeHg induced cytokine changes, relative to vehicle (Veh) only treated group, and 2. whether the combined PCB + MeHg or full Arctic chemical mixture promoted further cytokine elevations greater than their individual effects. In our second study, female litter-mates received the same treatments except that all rats were administered LPS on PND 208-212 in order to determine if the cytokine response to LPS was modified by previous perinatal exposure to the chemicals. Hence, the first study examined basal cytokine levels at PND 145-147, while the second study used litter-mates that were administered LPS 90 min before sacrifice at PND 208-212. Data were evaluated using a StatView (version 6.0) statistical software package available from the SAS Institute, Inc.”

11. Two separate studies are described – what is the difference between? Why do the authors describe the 5 groups of exposure for this “second study” in this section? I suggest to add more details about this experimentation in previous sections.

Response: These details have now been added to the results section.

P. 9 paragraph 2: “On PND 208-212, a separate set of female litter-mates were challenged with the bacterial endotoxin, lipopolysaccharide (LPS; 48 ug/kg; i.p.) and 90 minutes later were sacrificed by decapitation and the hypothalamus was dissected and frozen for subsequent cytokine analysis (see procedures below for dissection and assay details). We have previously found that similar endotoxin doses readily provoked behavioural, neurochemical and cytokine changes within hypothalamic and stressor-sensitive limbic regions (Anisman et al., 2009; Gibb et al., 2009; Hayley et al., 2008). Hence, we sought to assess whether early life exposure to the Northern toxin constituents would enhance the neuroinflammatory cytokine cascade that is provoked by LPS challenge. Indeed, this situation should mimic instances where, individuals are exposed to multiple environmental contaminants and subsequently encounter typical infectious agents.”
Results:

12. “The results about LPS change should be shown to readers. All the raw data of experiments reported as “data not shown” should be presented.”

Response: We now include all data from the second LPS injection study. Although these data did not reach statistical significance, they are presented in all the figures for comparison purposes.

13. I have impression that the second experiment was not designed to answer the research question, but available female animals from another study were used.

Response: We respectfully indicate that the reviewer’s impression is wrong. The animals were all from the same dosed litters from the same study and differences are related to the specific acute treatments in adulthood.

In addition, no rationale is given for the dose of LPS.

Response: We now justify the LPS dose in the results section. See point 11 above.

15. As only females were used and cytokines were analyzed in adult age, the question about hormonal cycle should be addressed: were they all ovariectomized? Does hormonal cycle change cytokine levels in hypothalamus? How this parameter was controlled?

Response: Animals were not ovariectomized; we would have definitely mentioned such a crucial detail if this was the case. To our knowledge there are virtually no data indicating that hormonal cycle would affect cytokine levels in the hypothalamus. We did find evidence that cytokines might vary within the reproductive organs with different stages of the hormonal cycle, but nothing indicating hypothalamic changes. This parameter was not controlled for two reasons: 1. most importantly, we wanted to mimic the actual human condition and not surgically or otherwise produce an abnormal animal and 2. it is unclear to us how this variable could be reliably controlled without having serious behavioral consequences upon the animals.

16. Also, authors should show target blood levels of contaminants and those found in exposed animals.

Response: The reviewer is requesting information that is not available for these specific animals. However, we have conducted previous studies using the same species, the same dosing mixture at the same doses, the same breeding and dosing methodology and have referenced the blood residue data available for that in Chu et al 2008. This has been more clearly indicated in the discussion.

17. In Discussion section, the affirmation that “these findings are consistent with the substantial evidence indicating that exposure...can influence central nervous system functioning” is speculative without any experimental evidence showing
how IL-6 and IL-10 can be involved in development of neurocognitive deficits similar to those observed in exposed subjects.

Response: This section of the discussion has been toned down and we try to provide a more balanced interpretation of the data. We thank the reviewer for pointing out this over-interpretation.

18. The Discussion should be focused on disturbed cytokines, the pathway they are involved, and the link between them and brain development and functioning. Also, the delayed effect should be addressed – how may it be explained? By persistence of exposure? By abnormal glia development?

Response: Once again, we have re-written a substantial portion of the discussion with a focus towards more fully integrating the cytokine data with previous findings and the relevant pathways involved. We specifically deal with the issue of persistence and potential glial involvement in several places in the discussion (particularly on pp 22-23 and 25-26).

19. How can we conclude on persistent changes by using the only one time point, the 120th postnatal day?

Response: This question really is dealing with the issue of persistence versus delayed effects. Persistence suggests effects that show up early and persist. Delayed effects are effects that just show up later and were not present earlier, possibly related to developmental stage, possibly related to time required to develop the effects (e.g., cancer). The reviewer is quite correct about this. For a health risk perspective, persistence and delayed effects are serious effects. We have addressed this in the discussion to clarify that we detected the effects long after dosing ceased but cannot discern whether effects are persistent or delayed effects.

20. The presentation of results should be more concise, no need to comment ANOVA and post hoc tests separately. No need to use terms like “omnibus ANOVAs”, “just missed significance” etc.

As Results are presented now, they are too descriptive for a scientific paper...

Response: We have attempted to change the wording of the results to be a little more concise. However, we respectively disagree with the reviewer on this point. Description of the ANOVAs and post hoc tests as presented in this manuscript are commonly done in the literature and we and many others have routinely taken this route in many different journals (e.g. J. Neurosci., Neurosci., Brain Beh. Immun., Eur. J. Neurosci......). Technically, one first assesses ANOVA significance before moving to post hoc or follow up tests.

21. Page 17, “Perinatal exposure…” – it would be more informative to give the % of increase of IL-6.

Response: We believe that it is always preferable to present raw values when
possible. The % increase can be easily estimated from raw values but the converse is never the case.

22. Discussion: The second affirmation should be followed by a reference to a published paper or to results of this study.

Response: No change to manuscript as we could not determine what the reviewer was actually referring to so we could not address this issue

23. References – please check the format for references

Response: References have be re-checked.

24. Figures – five figures should be grouped in one.

Response: We tried this but it was much too cumbersome and not at all conducive to communicating the data clearly.

25. Asterisk should be explained – ANOVA? p<0.05?

Response: Done in figures.

Response to Reviewer 2

1. Several previous studies used the NCM (nordic chemical mixture) as established in the cited paper by Chu et al., still the description of the preparation of the solution and the dosing reads cryptic.

Response: A better description of the preparation of the mixture has been added. We also identify some minor errors that have been corrected.

2. What I do not understand is the term high dose solution since only one dose was used. e.g.: "The dosing solution for the high dose of the full mixture...."

Response: Reference to a high dose in this study was an error and has been corrected.

3. I wonder why the authors did not refer to the other relevant citations in this setting:


Gene expression profiling in rat cerebellum following in utero and lactational

Response:
The pertinent papers have now been added to the manuscript. We thank the reviewer for attention to such important details.

I saw some minor spelling errors.
Response: re-checked

Response to Reviewer 3

1. There is emphasis on the possible contribution that environmental toxins, particularly the Arctic chemical mixture, play on cognitive neurological impairments, yet the only brain region examined was the hypothalamus. The authors indicate they ‘chose to focus on the hypothalamus given that this region has higher than normal levels of most cytokines and the fact that most research indicates a critical role of hypothalamic functioning in cytokine induced neuronal alterations’. While true, higher levels of brain cytokines are expressed in the hypothalamus that are thought to mediate many sickness responses such as fever, decreased food/water intake, HPA and sympathetic nervous system responses, cognitive impairment including learning/memory and attention deficits are typically not thought to be mediated by cytokines in the hypothalamus, but in brain areas such as the hippocampus and prefrontal cortex.

Response: We fully agree with the reviewer and have now added additional description in the discussion of the importance of the hypothalamus and cytokines in behavioral, sickness and stressor-like effect. Indeed, much of our own previous work deals with this very issue.

e.g. P. 20 discussion: “...For instance, gestational exposure to PCBs, mercury, lead, and organic pollutants have been associated with later cognitive disturbances in infants and children and may contribute to disorders of attention and activity (Stewart et al. 2003; Colosio et al., 2003; Steenland et al., 2000; Curtis & Patel, 2008). Yet, such cognitive effects are generally mediated by hippocampal and cortical brain regions, whereas hypothalamic brain changes (as observed in the present investigation) are typically associated with stress responses and hormonal output. Indeed, a plethora of data indicates that psychological and immunological (particularly LPS) stressors promote marked hypothalamic neurochemical alterations, often coupled with signs of sickness (e.g. fever, piloerection, ptosis, curled body posture) or depressive-like symptoms, such as anhedonia (Dantzer, 2006; Anisman et al., 2008; Hayley et al., 1999, 2008; Dunn, 2006; Godbout et al., 2005).”

2. Including additional brain areas would strengthen the paper and better allow the authors to discuss the possible role environmental toxins have in altering
cytokine production in brain areas that mediate higher order cognitive processes. If other brain areas were not dissected or cannot be included, then at least further discussion of the kinds of physiological responses that might be altered by cytokine changes in the hypothalamus might be warranted.

Response: Again, we agree with the reviewer that it would be great to include other brain regions. Unfortunately, these are not available. To this end, we have attempted to further highlight the importance of the hypothalamus in the discussion section.

3. The ‘mixture preparation’ section of methods needs clarifying.

Response: Mixture preparation methodology section has been revised and minor errors have been corrected.

4. For example the manuscript states, ‘The dosing solution for the high dose of the full mixture (5.0 mg/ml) was then prepared by combining 0.84 g of the OC stock solution, 0.37 g of the PCB stock solution, 0.34 g of the MeHg stock solution and 0.59 g of corn oil.’ Table 1 lists the concentration in micrograms per ml, not mg/ml.

Response: Concentrations are presented as ug/ml in Table 1 because presenting concentrations as mg/ml would be rather cumbersome and less readable because of the low concentrations of some chemicals (e.g., aldrin concentration would be 0.000065 mg/ml. might change Table 1 units to ug/ul which would make more sense since dosing volume is in ul/gm).

5. Also, when combining the stock solutions it seems odd to report a weight instead of a volume, and it would take 15.85 liters of OC stock solution (reported to be at a final concentration of 0.053 mg/ml) to get 0.84g. Overall, it is unclear how the calculations result in the reported concentrations.

Response: Mixture preparation section in the methods has been revised.

6. Results (first paragraph): Include F values (df) for basal IL-1 in results

Response: F values have been added.

Also, p=0.07 is listed after ‘LPS treatment’ but do the authors mean the basal levels presented in Figure 1 as inferred by the post-hocs analyses?

Response: As now stated in the results section, the p=0.07 refers to the ANOVA. In accordance with comments from reviewer 1, we have toned down our interpretation of the data and use of post hocs, instead simply mentioning the trend towards elevated IL-1b levels. The LPS treated rats showed no significant differences as a function of the perinatal chemical treatments.

Results (last paragraph): Include ‘Figure 4-5’ in description

Response: This has now been corrected.
Methods: What was the brain tissue homogenized in?

Response: All tissues were homogenized in plastic centrifuge tubes using our standard multiplex diluent mixture (Upstate Beadlyte proprietary buffer solution).

10. Discussion (first paragraph): ‘exposure to realistic levels of environmental chemicals provoked long-term inflammatory cytokines’. Equating concentrations of drugs across species is always a tricky issue as the absorbance, metabolism, clearance of the drugs may be different. While it’s nice the authors took the time to determine a ‘realistic’ level of toxin to administer, some discussion of the precautions/limits should be discussed as well.

Response: Have clarified that we have done previous work showing that this mixture and dosing regimen produce maternal rat blood levels comparable to human maternal blood levels. Still, we include sentence of caution about limitations of extrapolating between species.

11. Discussion (paragraph three): Authors may not want to abbreviate CNS since this is not a brain journal.

Response: This has been corrected.

Minor issues not for publication:

1) Abstract (Methods): ‘cytokine levels were measured withy a suspension-based array system and differences were determined using ANOVA’

2) Methods (first sentence): ‘nulliparious’ is spelled nulliparous

3) Methods (Mixture preparation section): ‘because it is not lipophilic and it’s contribution to the mixture’; no apostrophe is needed

4) Methods (Mixture preparation section next sentence): “Because a number of the chemical had extreme values”; chemical should be plural

5) Methods (mixture preparation; second paragraph): “This PCB stock solution was then transferred”; change to ‘was’

6) Methods (statistical analyses): “was modified by the perinatal chemical exposure”; should be perinatal

7) Methods (statistical analyses): “animals also received the adult LPS injection”; reword to indicate animals received LPS injections when they were adults

Results (last paragraph): ‘post hoc analyses based on out a priori’; should be ‘our’

These “minor” corrections have been made in the manuscript. The authors sincerely thank the reviewers for attention to such details. We believe these changes definitely strengthen the manuscript.