Author’s response to reviews

Title: Nitrosamine exposure exacerbates high fat diet-mediated neurodegeneration

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Response to Reviewer’s reports

We have taken into consideration all of the comments and critiques made by these thoughtful reviewers, and have addressed all matters of concern. Where appropriate, we have included additional data and clarifications as requested, and a revised manuscript with changes marked in red font is submitted to facilitate review of the changes. In addition, for clarity, we have included a Materials section to indicate sources of reagents used in the research.

One opinion raised by two of the referees was that cognitive behavioral study data might be included in the manuscript. Although we have done such studies and now provide some data in a supplementary table for review only, we feel strongly that the inclusion of such data would move the focus of the manuscript off target. The primary objective of this manuscript is to examine molecular and biochemical parameters of neurodegeneration in relation to combined effects of an environmental toxin exposure and excessive calorie intake. This is a novel concept. Our study was designed to help explain the current overlapping epidemics of diabetes and neurodegeneration, since both insulin resistance disease processes exploded in concert over the past 3 decades. In a future publication, we hope to assemble a systematic and detailed characterization of cognitive-behavioral deficits that develop with progression of neurodegeneration mediated by obesogenic diets and environmental toxin exposures.

Our point-by-point responses are provided below. We believe that our responses and revisions adequately address all queries, and hope that our revised manuscript is now acceptable for publication in BMC-Endocrine Disorders.

Reviewer: Cedazo-Minguez

We agree that brain regions studied should include targets of neurodegeneration. Our reason for studying the cerebellum is that there is good evidence that the cerebellum is affected in AD, as well as in other neurodegenerative diseases. In this regard, articles by Larner (1997), Wegiel, et al (1999), Cole, et al (1993), and
Ishii, et al (1997) demonstrated that, although the cerebellum is relatively spared in AD, it is in fact a target of neurodegeneration. We also previously showed that in humans with AD, the cerebellum exhibits insulin resistance and other biochemical and molecular indices of neurodegeneration (Steen, et al 2005). An additional reason for selecting the cerebellum for study is that this brain region is highly insulin and IGF responsive and convenient for examining long-term effects of insulin resistance using morphological and molecular approaches. Finally, by focusing on one brain region, we were able to assemble a digestible manuscript that deals with a novel concept, i.e. the potential additive or synergistic effects of double-hits, i.e. nitrosamine plus HFD exposures on neurodegeneration. We have incorporated comments along these lines into the Discussion.

The chow diets were purchased from Research Diets, Inc. We have included the breakdown information on protein, carbohydrate, fat, and cholesterol content in the meal as percentages of kcal in the Methods section.

We have cited evidence that chronic high fat intake leads to mild neurodegeneration.

Statistics—perform repeated measures ANOVA with multiple comparisons. We have followed this recommendation and incorporated results of all inter-group comparisons into the Results section and figure and table legends.

Antibody information—We have provided information about the source and characteristics of the phospho-specific antibodies, as well as all immunological reagents.

Immunostaining studies of relevant regions. We justified studying the cerebellum. The cellular distribution of immunoreactivity corresponds to both neurons and glia.

Response to Reviewer: Cheng Juei-Tang

The inclusion of cognitive-behavioral studies in this manuscript would go beyond the scope of the intended investigations, which were focused on characterizing mechanisms of neurodegeneration and brain insulin/IGF resistance. We employed multiple assays to demonstrate the neuropathology as well as significant abnormalities in gene expression and insulin/IGF signaling in the brain. The manuscript is already quite long, and we feel the additional suggested studies are better suited for a different audience. However, to satisfy the curiosity of this reviewer, we have included a supplementary table showing that the rats were cognitively impaired based on Morris Water Maze tests. We do not wish to include this data in the present manuscript because the additional details needed to integrate everything would damage the focus of this already large body of work.

The analyses of the insulin, IGF and IRS molecules were based in qRT-PCR because we consistently achieve excellent agreement between the mRNA and protein studies. The methods of data acquisition and analysis are clearly indicated in the text and legends.
We have discussed the paradoxically reduced peripheral blood lipid profiles observed with chronic HFD feeding. This phenomenon has been reported previously and presumably is due to sequestration of lipids into tissues such as adipose tissue and liver [Page 11].

Plasma insulin levels are included in Table 2 and first paragraph of Results. We used ELISAs to measure immunoreactivity as shown in Figures 2 and 3.

Our investigations were focused on determining mechanisms of neurodegeneration and brain insulin/IGF resistance. We employed multiple assays to demonstrate significant effects on multiple levels. The manuscript is already quite long, and we feel the additional suggested studies would be better suited for a different audience.

Rats were administered NDEA in low doses and in 3 injections. Rats were maintained on HFD or LFD for 8 weeks. Their total period of study was 12 weeks. These details are stated in the Methods sections and re-emphasized in the Discussion [Pages 16-17].

Response to Reviewer: Katsuo Kamata

Insulin levels have been added to Table 2 and described in the first paragraph of Results.

We have discussed the paradoxically reduced peripheral blood lipid profiles observed with chronic HFD feeding. This phenomenon has been reported previously and presumably is due to sequestration of lipids into tissues such as adipose tissue and liver [Page 11].

ROS formation—In addition to the 4-HNE assay results included in the original manuscript, we have added data concerning MDA, nitrotyrosine, and protein carbonylation assay results [Figure 3; Methods; Results-page 14].

As suggested, we measured serum adiponectin and leptin, in addition to brain TNF-alpha, IL-6 and IL-1b levels. Those results are shown in Table 2 and/or reported in the Results section [Pages 14-15].

Response to Reviewer: Chang-Ju Kim

We have included a brief discussion of the evidence that obesity and chronic HFD feeding cause neurodegeneration. There is also data showing that peripheral insulin resistance/diabetes is linked to neurodegeneration. [Page 6].

The inclusion of cognitive-behavioral studies in this manuscript would go beyond the scope of the intended investigations, which were focused on characterizing mechanisms of neurodegeneration and brain insulin/IGF resistance. We employed multiple assays to demonstrate the neuropathology as well as significant abnormalities in gene expression and insulin/IGF signaling in the brain. The manuscript is already quite long, and we feel the additional suggested studies are better suited for a different audience. However, to satisfy the curiosity of this reviewer, we have included a supplementary table showing that the rats were cognitively impaired based on Morris Water Maze tests. We do not wish to
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