Author's response to reviews

Title: Glutamatergic Deficits and Parvalbumin-Containing Inhibitory Neurons in the Prefrontal Cortex in Schizophrenia

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Author's response to reviews: see over
Dear Dr. Alam,

We are re-submitting our manuscript entitled: “Glutamatergic Deficits and Parvalbumin-Containing Inhibitory Neurons in the Prefrontal Cortex in Schizophrenia” that we would like you to once again consider for publication in BMC Psychiatry. We have carefully reviewed the comments made by the three reviewers and have made changes in the paper in accordance with their recommendations. Changes made are in italic in the revised manuscript. Below we address the specific comments made by the reviewers in a point-by-point manner.

Comment: Human brain tissues were used in this study, but no statement regarding appropriate informed consent was stated in the manuscript.

Response: Postmortem human brain research is not considered human subject research and hence informed consent is not required. However, for each brain that has been donated to the Harvard Brain Tissue Resource Center, informed consent has been obtained from the next of kin. This process is under the oversight of the McLean Hospital Human Subject Research Committee. This has been made clear in the text (page 4).

Reviewer 1

Comment 1: Table 1 provides the demographic information about brain samples. Could you provide RIN (RNA Integrity Number) score (how and when RIN was measured)? Although it is a valuable index, RIN is very important for mRNA quality/stability in postmortem brains.

Response: We do not have RIN information for the samples. This would have required the additional procedure of extracting RNA from the samples. RIN has been shown to be a good indicator of global RNA quality for gene expression profiling experiments, where thousands of genes are being studied at one time. In in situ hybridization experiments, such as the one reported here, the focus is on one or two specific gene transcripts. In this study, as the transcripts of interest (i.e. PV and NR2A) were clearly detected by our riboprobes, RIN is irrelevant. In fact, in any experiments, a good or bad RIN would not tell us anything about the integrity of a specific transcript, which can only be ascertained by directly measuring or quantifying the transcript, such as by qPCR or in situ hybridization.

Comment 2: Matching samples is not so perfect, for instance, 7 females in control group (n=20), and 11 females in case group (n=20). You did the analysis for confounding variables using Pearson's correlation. Did you try ANCOVA, considering RIN, sex, age, PH, PMI as covariances?
Response: We had performed ANCOVA but found no significant differences in our data. See Confounding Variables subsection, page 9.

Comment 3: Did you conduct normalization for mRNA quantification, for example, using reference gene(s)/housekeeping gene(s)? Also, confirmation of the expression levels of NR2A mRNA and PV mRNA by quantitative real-time RT-PCR will make the conclusion solid.

Response: We are unsure as to what the reviewer is referring to regarding normalization in in situ hybridization experiments. We would be happy to address it if this can be clarified for us. We agree that the results of this study will need to be replicated. In theory, this can be done by the approach proposed by the reviewer. However, this will require isolation of PV neurons, e.g. using laser capture microdissection, from each of the cortical layers, and measuring NR2A expression in these neurons, e.g. using qPCR. Doing this in a sufficient number of cases (e.g. N=10 in each group) will involve a tremendous amount of work and will be very costly. We believe that, in the future, replicating the reported findings in a different cohort of cases using the same approach will be very important.

Comment 4: In "Density of PV mRNA+/NR2A mRNA+ Neurons" of "Results" section, the word "highly" should removed from the sentence "The effect of diagnosis was highly significant (F = 6.62; df = 1,38; P= 0.01).", because P=0.01 is significant not highly significant. In the sentence "Furthermore, this effect was layer specific (F = 3.75; df = 1, 38; P = .006)." , it's better to change "P = .006" to "P = 0.006", for context continuity. In addition, the cutoff of statistical significance must be pointed out.

Response: The word “highly” significant has now been removed. In addition, notation of statistical data has been amended for text continuity. Finally, the cut off point for statistical significance is now clearly stated in the manuscript: “statistical tests were conducted with $\alpha = 0.05$.” See Statistical Analysis subsection, page 9.

Reviewer 2

Comment 1: The authors missed scale bar in Figure 4 (A).

Response: The manuscript now includes a scale bar for the figure in question.

Reviewer 3

Comment 1: In the abstract, the BACKGROUND part seems lack of information of vGluT1. The background should correspond with the following experiments and conclusions.

Response: We have modified the text in the Background section of the Abstract to correspond with the experiments and conclusions.

Comment 2: Page4. BACKGROUND part, sentences beginning form” In this study, using………” to the end sentence of this paragraph, belong to the parts of methods, results and conclusions, It’s better to use other expression to show why the authors designed this experiments. The authors should focus on the reasonability of their experiments rather than conclusions.

Response: In the Background section, we establish the context and the “reasonability” of the work being reported. In addition, we very briefly summarize the approach we used to address the questions of interests and the findings. This is a format that is often employed by many scientists in the field.

Comment 3: In the results, Figure 4. Why the authors only show the results of layer 3 and layer 5, I’m curious about the layer 4 result.
Response: As has been demonstrated by one of us (T. Kaneko), vGluT1 is selectively localized to terminals of corticocortical glutamatergic axons and hence vGluT1 immunoreactivity is quite weak in layer 4, which receives thalamocortical projections. On the contrary, terminals of thalamocortical axons express vGluT2, but not vGluT1. In fact, we are currently examining the possible changes of vGluT2-immunoreactive terminals in layer 4 in schizophrenia.

Comment 4: Page 10. “Cellular Expression of NR2A mRNA”, the expression of NR2A mRNA in the PV cells seems no changeable in schizophrenic patients. However, page 11, last sentence “… in a subset of PV neurons, the expression of NR2A is reduced to a level that is no longer experimentally detectable”. I’m confused with these two expressions. Please explain if possible.

Response: The finding that the density of PV+/NR2A+ double labeled neurons is decreased suggests that in a subset of PV cells, the expression of NR2A has become undetectable in schizophrenia. However, in those PV cells that express NR2A, the level of NR2A expression is unchanged.

Comment 5: Discussion part. Page 12, in the second paragraph, the authors mentioned that a single polymorphism in neuregulin-1 gene is associated with vGluT1 expression. The evidence presented here doesn’t increase the power of explanation for the inconsistent results of this paper and others’.

Response: While the evidence presented here may not provide a direct link or explain the inconsistent results of this paper and other research in the field, we think that it is still interesting to highlight the point that deficits in vGluT1 in both striatal and hippocampal regions in addition to vGluT1 density in patients with schizophrenia have been associated with neuregulin-1, a prominent candidate gene for schizophrenia.

Comment 6: References in this manuscript should be reduced.

Response: We have reduced the number of references.

We hope that we have satisfactorily addressed the comments raised by the reviewers and that you find this manuscript suitable to be published in BMC Psychiatry. We thank you for your consideration.

Sincerely,

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