Author’s response to reviews

Title: Association between NRGN Gene Polymorphism and Resting-State Hippocampal Functional Connectivity in Schizophrenia

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Version: 1 Date: 13 Nov 2018

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Technical Comments:

Title page: Please include the email addresses for all authors on the title page. The corresponding author should still be indicated. Please also ensure these email addresses match the email addresses provided in the editorial manager system.

We added the email addresses for all authors on the title page as follows: ivanhey825@163.com, gongxh@fudan.edu.cn, yzykls@163.com, 13804071019@163.com, yangjian@cmu.edu.cn, Kyleonaz@163.com, zhou060117@126.com, jxw024@163.com, weisn1982@126.com. (Title page, line 7, page, 2)
Funding: Please state clearly the role the funder(s) had in your study in the "funding" section of the declarations.

We clarified the role the funders had in our study in the "funding" section of the declarations as follows: The funders had no influence on the research design, data collection, data analysis, interpretation of results, writing the manuscript or submitting for publication. (Funding section, line 17, page16)

Reviewer reports:

Marcos Santoro (Reviewer 1): 1- Page 4, line 42. This GWAS is quite old. It would be recommended the use of the last GWAS for schizophrenia published in 2014 (Nature). Please check if this SNP is still associated with schizophrenia.

We appreciate the reviewer’s comments. Although the last GWAS for schizophrenia did not report genome-wide significance for rs12807809, an expression quantitative trait loci (eQTL) analysis indicated an increased risk of schizophrenia with this SNP at a molecular level [1]. Furthermore, recent studies have examined this SNP extensively and implicated its involvement in structural and functional brain abnormalities in SZ as well as its association with symptom severity in this condition [2-8]. Given the clear importance of this SNP, we selected rs12807809 for this analysis.

2- The article needs some language improvements. In page 5 lines 20-29 the phrase seems to be copied from another article.

We apologise for the language issues. We have modified the concerning text as follows: This protein is called neurogranin (Ng), given that its immunoreactivity is correlated with granule-like structures in hippocampal pyramidal cells in electron micrographs. (Background section, line 15, page 4)
3- Page 10 line 50. The author should include the statistical results and not only the p-value. There is a clearly difference between the number of male/female between cases and controls (27% males in cases and 42% males in controls) this could affect the neuroimaging results.

We appreciate the reviewer’s thorough reading of our manuscript. We have included the statistical results and p-values in Table 1. We have further clarified this information in the main text as follows: There was no significant difference in age ($F_{3,154} = 0.137, p = 0.712$) between the diagnostic and genotype groups. Similarly, there were no significant sex differences between the diagnostic groups ($\chi^2 = 3.727, p = 0.054$), between the genotype groups ($\chi^2 = 3.343, p = 0.067$), or between the genotype groups within each diagnostic group ($\chi^2 = 2.054, p = 0.152$ in the HC group while $\chi^2 = 1.193, p = 0.275$ in the SZ group). There was also no significant difference in the duration of illness between the genotype groups within the SZ group ($t = -0.286, p = 0.776$) (Table 1) (result section, line 14, page 10). Considering the potential effect of sex on results, the statistical test we used in the original article was analysis of variance with sex as a covariate (line 4, page 10).

4- Page 10 line 50. Curiously, the number of female cases are higher than male cases which is very unusual for schizophrenia, the author should explain this difference.

We agree that this may seem unusual. In recruiting subjects, there was no restriction based on sex; the current sample represents all those who were recruited for the study. In similar studies, the number of female patients with schizophrenia was higher than that of male patients [4, 9-11].

5- The authors should include the results when considering each genotype individually before merging the C-carriers (eg. CC x CT x TT) or use a reasonable explanation to consider only the C-carriers x TT comparison (eg. TT genotype have a NRGN loss of function protein).

We agree with the reviewer that this clarification is important. We have clarified the explanation in the revised manuscript as follows: Large studies [5, 8, 12] found a low frequency of the CC genotype (less than 10% in both patients with SZ and HCs). In our study, the frequency of the CC genotype was 1.7% in patients with SZ and 12.1% in HCs. Furthermore, individuals with the TT genotype may have a higher risk of SZ than those with the C allele. Thus, we pooled participants carrying at least one C allele and compared them with T-allele homozygotes. (Method section, line 9, page 7)
6- Page 9 lines 7-9. How many individuals were excluded? This data should be addressed in the results section.

We have clarified the number of participants excluded and the reasons for exclusion in the revised manuscript: We excluded two patients with SZ and two HCs because of excessive head motion. (Method section, line 20, page 8)

7- Figure 2 b should be reformulated. It is not clear what this figure means. The authors only state that the TT cases have the lower FC values compared to other groups, but it is not clear which statistical test were used. Please include the p-values and other statistical measures in the plot. In this case a barplot would be easier to understand the results. Besides, the standard deviation is extremely high, did the authors check if there were any outliers?

We apologise for the lack of clarity and appreciate the reviewer’s careful reading of our manuscript. We have modified the figure and included additional statistical measures to better clarify our results as follows: Figure 2: Diagnosis by genotype interaction. (a) Significant regions of diagnosis by genotype interaction from two-way ANCOVA include the bilateral middle cingulate gyri and left anterior cingulate gyrus (P < 0.05 by GRF correction and 22 voxels minimum). The coloured bar represents the range of F values. (b) Shown here are the mean (standard deviation) FC values extracted from significant regions of the diagnosis by genotype interaction. Post hoc two-sample t-tests show that TT homozygotes with SZ have significantly lower FC values than do healthy TT homozygotes and C-carriers with SZ. *,p<0.05. (line 6, page 21) An improved figure was provided in revised_Figure2.docx. We checked to ensure the absence of outliers by means of plus or minus 3× standard deviations.

8- Page 12 line 23. These results that the TT genotype is associated with abnormal hippocampal function and not the T allele.

We agree that this distinction should be made for clear, accurate interpretation of our findings. We have modified the text in the revised manuscript as follows: These results suggest that the rs12807809 TT genotype in the NRGN gene is associated with abnormal hippocampal FC at rest in SZ. (Discussion section, line 6, page 12)
Gabriela Xavier (Reviewer 2): The manuscript aims to examine the association between a SNP in NRHN gene and hippocampal function in schizophrenia patients. The authors found interesting results of fMRI, apparently capable of distinguishing HC and SCZ groups. However, the genetic association is weak.

The main concern is about the statistic test the authors use. They opted for using ANCOVA test for evaluating the association among SNP genotyping, the disorder and functional connectivity. However, they do not show neither the results table, neither if their data present the assumptions needed for performing the test. From a weak statistical basis, the authors suggest the influence of the genotype on the other variables, which reduces the confidence of the findings. I highly recommend the authors to perform a more robust statistical test (as linear regression, for example) and evaluate the main effect of the genotype, of the group and their interaction with the functionality (dependent variable). It is really important to report the statistic results in a table or even in the body of the manuscript.

We appreciate the reviewer’s comments and agree with the use of robust statistical tests in studies. Because our study is a factorial experiment with two factors (diagnostic group and genotype) and two levels (diagnostic group (SZ/HC) and genotype (TT homozygote/C-carrier)), we chose two-way analysis of covariance rather than linear regression to investigate the effect of the SNP on resting-state functional connectivity (FC) of the hippocampus in a voxel-wise manner in the whole brain in patients with schizophrenia and healthy controls. Similarly, Lippard et al. divided all subjects (patients and healthy controls) into four groups based on diagnostic groups and genotype factors. Diagnostic group by genotype factorial models covarying age and sex, with grey matter volume or fractional anisotropy values as dependent variables, were used to assess non-hypothesised regions showing a significant diagnostic group by genotype interaction in the whole brain[13]. The results of two-way ANCOVA are reported in Table 2. FC values in the regions showing significant main effect of the diagnostic group and diagnostic group by genotype interaction were extracted, and linear regression was performed with the diagnostic group, genotype and their interaction as independent variables. FC values in the regions showing significant main effects of the diagnostic group and diagnostic group by genotype interaction were used as dependent variables to verify ANCOVA results. The linear regression results, which are reported in Table S2 in supplementary materials, were almost the same as the results of ANCOVA.

Another important issue to be reviewed (or at least better explained in the introduction) is the reason the rs12807809 was chosen for this analysis. As I understood from the introduction, the SNP is located in a haplotype, right? Is this SNP a tag SNP? If you have done the sequencing of
this region, why not to test all SNPs from the haplotype? Also regarding to genetic information, the authors say that the allele frequencies were in Hardy-Weinberg equilibrium and report the minor allele frequency. An allele frequency table would be very informative. Information about genetic ancestry of the participants would also be relevant.

These are important questions. We have further clarified the reason in the revised manuscript as follows: In recent years, in genome-wide association studies, a single-nucleotide polymorphism (SNP), rs12807809 in the NRGN gene was reportedly associated with SZ [14]. An association of rs12807809 with SZ was also found in 1005 SZ patients and 1069 controls in a South Indian population; further analysis found a moderate association of rs12807809 with flat affect and hallucinations [8]. In addition, an expression quantitative trait loci (eQTL) analysis provide evidence for an increased risk for SZ with rs12807809 at a molecular level [1]. Over the years, rs12807809 has been extensively studied; these studies suggested that rs12807809 was associated with structural and functional abnormalities in the brain as well as the severity of symptoms in patients with SZ [2-7]. (Background section, line 21, page 3) Because of the importance of the SNP, we chose it for this analysis. This SNP was identified in a haplotype block with rs12278912, and the rs12807809–rs12278912 haplotype was associated with schizophrenia in a Japanese population [15]. We did not sequence the gene or perform haplotype analysis. We selected rs12807809 based on previous studies indicating its contribution to susceptibility to SZ. We have provided an allele frequency table (Table S1) in the revised manuscript. We included the ethnicity of participants as follows: Most participants were Han Chinese (n=134, 85% of total participants; n=52, 88% of patients with SZ; n=82, 83% of HCs), and there was no difference in the percentage of Han Chinese between patients and HCs. (Method section, line 19, page 6)

As a minor concern, I suggest the authors to add information about the Papez circuit in the introduction, once it is involved in one of the main conclusions of the study.

We appreciate the reviewer’s suggestions. We have added information about the Papez circuit as suggested in the revised manuscript as follows: The hippocampus is a major component of the brain and plays important roles in learning and memory. Additionally, the hippocampus is the beginning and end of the Papez circuit [16] and an important component of the limbic system. (Background section, line 7, page 5)
Reference:


