Author's response to reviews

Title: No Relationship between 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase and Schizophrenia in the Chinese Han Population: an Expression Study and Meta-analysis

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Author's response to reviews: see over
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Dear the Editor,

I would be grateful if you could kindly consider to have our revised manuscript of “No relationship between 2’,3’-Cyclic nucleotide 3’-phosphodiesterase and schizophrenia in the Chinese Han population: an expression study and meta-analysis” published in the category of regular research article in your journal. There is no financial interest. And I have the permission giving by all the authors. All the consent of patient in form of a signature can be received of a recognizable patient and kept by us. We would like to declare that this manuscript has not been, and will not be submitted elsewhere for publication before the decision made by you.

We look forward to hearing from you soon.

Yours sincerely,

[Signature]
The following are the response to reviewers’ comments.

Reviewer’s report 1
Title: No Relationship between 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase and Schizophrenia in the Chinese Han Population: an Expression Study and Meta-analysis
Version: 1 Date: 26 January 2009
Reviewer: Esa Veli Juhani Leinonen

1. The number of patients and controls is rather small and in the subgroups even smaller. Thus the analyses of subtypes of schizophrenia as well as of different antipsychotics may not be relevant. The gender distribution between patients and controls is different. Should this be stated in limitations.

Answer: Thank you for your advice. Sometimes it is not easy to get enough PBL samples and proper demography of them. So as you suggested, I add these limitations in the pg. 17, "we should be caution to draw this conclusion because the samples size is relatively small. And the different gender distribution between patients and controls would be addressed as another limitation."

2. A limitation is also that no formal diagnostic interview method (e.g. SCID) was used when assessing the patients.

Answer: A thoughtful and professional question! As stated in pg. 5, "Cases and controls underwent a clinical interview administered by two independent senior psychiatrists, based on DSM-IV (American Psychiatric Association). All patients received a DSM-IV diagnosis of schizophrenia with the following sub-diagnosis: undifferentiated (n=62), paranoid (n=18), disorganized (n=3), catatonia (n=2), residual (n=1). "The diagnostic method in our study is DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, version IV) till now we did not have SCID system. DSM-IV is well-accepted criteria and can be applied to classify sub-diagnosis groups. I am sure if we have SCID (Structured Clinical Interview for DSM-IV), it would be better.

3. The selection criteria of the papers for meta-analysis are obscure. These should be clarified.

Answer: As stated in pg. 11, " Eligible studies had to meet the following criteria: (1) were published in peer-reviewed journals, (2) were independent studies using original data, (3) provided sufficient data to calculate the odds ratio (OR) with confidence interval (CI) and P-value, (4) were case-control association studies investigating CNP polymorphisms, (5) described the relevant genotyping primers, machines and protocols or provided reference to them, (6) diagnosed schizophrenic patients according to DSM-IV criteria, (7) used healthy individuals as controls. We searched PubMed citations (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) up to January 2009 using keywords "CNP" and "schizophrenia"."

According to the above criteria, we collected 4 independent studies for rs2070106 and 3 independent studies for rs8078650, including the present studies.

Minor:
1. Shanghai Institute of Mental Health is given in affiliations but none of the authors is belonging there?
Answers: Thanks for your carefulness and patience. I have deleted this affiliation.

2. pg. 2: "Peirce et al. identified......" In which population?
Answers: As stated in pg. 2, Peirce et al. identified significant association between the exonic SNP rs2070106 and CNP expression (P<0.001) and lower expression levels of the A allele (P=0.04) in the white subjects from the United Kingdom and Ireland.

3. pg. 4: Were the controls also somehow evaluated?
Answers: As stated in pg. 5, "Cases and controls underwent a clinical interview administered by two independent senior psychiatrists, based on DSM-IV (American Psychiatric Association)." None of controls had a history of psychotic disorders.

4. pg. 4: How were the patients selected to different antipsychotic medications?
Answers: According to the hospital case notes, the antipsychotic medications were selected on the basis of individuals' previous experience of the drug.

5. pg. 4: Which were the five SNPs included in this study? (Should be given here).
Answers: As you suggested, I add "we genotyped five SNPs (rs4796750, rs8078650, rs2070106 rs11079028 and rs4796751) and performed..."

6. pg. 7: On my mind Fig 1. is not very informative and could be excluded (except part 1c).
Answers: I think part 1a provides a clear and direct picture of the genomic structure of the CNP gene locus and the locations of SNPs we investigated in our study. Part 1b is the estimation of LD structure of these SNPs. I applied the Haploview software to calculate the LD and haplotype distribution. So, these pictures may give some information to a certain degree or can be put into supplementary files.

7. pg. 7: What do the authors mean "...... the expression of CNP was reduced by 10 % in 79 schizophrenics......? Do they mean at least 10 % reduction in 79/86 schizophrenics and 84/94 controls, respectively? Fig 2 is also not important, all the negative data can be given in the text.
Answers: It means "at least 10 % reduction in 79 schizophrenics compared to 84 controls." We performed the quantitative real time PCR using 86 schizophrenia patients and 94 controls. "Some results were abandoned due to the deviation from 4 replications. We got valid expression data of 79 schizophrenics and 84 controls." I made the changes in results pg. 2 for this unclear statement. I think we could see the results from the figure more clearly and directly or it could be saved in supplementary files.

8. pg. 7: Were all the same SNPs studied also in the other reports included in the meta-analysis as in the present study?
Answers: To the best of our knowledge, this is the first meta-analysis report of CNP. All the case-control association studies investigated rs2070106 because of the previous positive result in the white samples. And rs8078650 was included in the Asian samples as a result of the different allele frequency among different ethnic populations.

9. pg. 8: CEU is missing in the list of abbreviations.
Answers: As you suggested, I add "population of western European ancestry (CEU)" in the discussion pg. 1.

10. pg. 8: sentence "Previous reports suggest altered expression of genes associated with myelinisation..... " remains unclear to me.
Answers: I changed it as "Previous reports in schizophrenia suggest altered expression of genes associated with myelination of neurons in peripheral blood lymphocytes was consistent with results from
postmortem brain tissue studies."

11. pg. 8: "Our result are lower by 10 %...... Compared to what?

**Answers:** I changed it as "Our results suggested expression levels of CNP in schizophrenic patients are lower by 10% compared to unaffected controls but with no significant difference."

12. pg. 9: "However, our study detected no difference between females and males... Probably this was also the case in the patients?

**Answers:** Our own study detected no difference between females and males either in healthy individuals, in the patients or in the combined samples.

**Reviewer's report 2**

Title: No Relationship between 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase and Schizophrenia in the Chinese Han Population: an Expression Study and Meta-analysis

Version: 1 Date: 3 January 2009

Reviewer: Albert Wong

1) There should be a brief review of the known function of the CNP gene and protein in relation to oligodendrocyte function and schizophrenia. In particular, the authors should present support for their hypothesis that genetic variation or altered expression levels of this gene can produce the clinical features of the disease. I.e. More mechanistic consideration of how changes at the genetic or transcription level for CNP are involved in the pathophysiology or etiology of schizophrenia.

**Answer:** Thanks for your advice. I add the function of CNP gene in the background pg. 2. "2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) is used as a marker protein of myelin-forming glial cells. In brain development, CNP is distributed in cells of the oligodendrocyte lineage and is maintained throughout life." Although the precise function of CNP in oligodendrocytes is unclear, recent evidence suggests that it interacts with mitochondria and cytoskeletal proteins and may promote microtubule assembly or act as a membrane anchor for tubulin.

Also, I add the possible relationship that myelination and oligodendrocyte function may be involved in the schizophrenia mechanism in pg. 1. "Myelination and factors that affect myelination, such as the function of oligodendroglia, are critical processes that could profoundly affect neuronal connectivity, especially given the diffuse distribution of oligodendrocytes and the widespread distribution of brain regions that have been implicated in schizophrenia."

In Background pg. 3, the transgenic mouse model experiments indicated CNP-deficient mice showed features (a reduction in overall brain size, enlarged ventricles and corpus callosum atrophy) which may also be implicated in schizophrenia. In pg. 2, "Lower expression levels of CNP have been detected in the postmortem brains of schizophrenic patients." "Peirce et al. identified significant association between the exonic SNP rs2070106 and CNP expression (P<0.001) and lower expression levels of the A allele (P=0.04)." These evidences provide support for the association between genetic variation or reduced expression of CNP and schizophrenia. Since CNP is thought to be important for oligodendrocyte function and myelination, altered expression of CNP has been interpreted as supportive of the hypothesis that
altered oligodendrocyte function and myelination may be an etiological factor in schizophrenia.

2) I do not agree that the literature cited by the authors supports the notion that the **CNP** gene is "one of the most promising candidate genes for schizophrenia [abstract line 1 and background paragraph 2]." While the quoted statement is not precise (how many genes would be considered "most promising": 10 or 100?), the subjective tone is certainly strong, but in my opinion, overstated. For example, only one genetic linkage study pointing to the genomic region in which **CNP** is located is cited, without mention of the many other linkage studies that highlight other chromosomal regions being linked to schizophrenia. There are four cited post-mortem studies of altered **CNP** expression in schizophrenia, but no mention of the large body of post-mortem work on other altered transcript or protein levels in schizophrenia, nor a discussion of how prominent or obscure **CNP** is in genome-wide transcriptomic or proteomic screens (i.e. how does the change in **CNP** gene product compare with other genes?).

**Answer:** Thank you for your preciseness in wording, my statement is not appropriate. As you suggested, I deleted "most" in the abstract: "2',3'-cyclic nucleotide 3'-phosphodiesterase (**CNP**), one of the promising candidate genes for schizophrenia,..." and made some changes in the background.

In Background pg. 2, I complement some details about the post-mortem work as supports "Lower expression levels of **CNP** have been detected in the postmortem brains of schizophrenic patients[3, 7, 9]. Hakak et al. used the expression microarray in postmortem dorsolateral prefrontal cortex of schizophrenic and control and detected notable differential expression of myelination-related genes suggesting a disruption oligodendrocyte function in schizophrenia[3]. Tkachev et al. found schizophrenia and bipolar brains showed down-regulation of key oligodendrocyte and myelination genes, including transcription factors that regulate these genes, compared with control brains[9]. In post-mortem studies of the anterior frontal cortex Flynn et al. found lower immunoreactivity of protein encoded by the **CNP** gene in schizophrenia patients (P=0.05)[10]. In a case-control study Peirce et al. identified significant association between the exonic SNP rs2070106 and **CNP** expression (P<0.001) and lower expression levels of the A allele (P=0.04) in the white subjects from the United Kingdom and Ireland [11]."

3) There is no power calculation. Especially with the small sample size, it is important to know the threshold at which this study might show statistically significant genetic associations. The failure to replicate previous genetic association results could stem from the low power of this study. In addition, there is only a cursory mention of the three papers considered for the meta-analysis, even though this current paper represents an attempt to replicate those results. Again, there is little discussion of what the genetic association data means in terms of disease mechanisms.

**Answer:** Instead of genetic association study, our aim to genotype the sample is to correlate the individual genotype with the expression data, to see if there is relationship between a certain genotype and schizophrenia. As previous paper has investigated the case-control association on the Beijing Chinese population, our frequency data were highly consistent with their results. To make it more convincing, we used meta-analysis of 3930 samples to increase the power. The large sample size is necessary to obtain more conclusive association results.

As stated in Methods, pg. 7, " Eligible studies had to meet all of the following criteria: (1) were published in peer-reviewed journals, (2) were independent studies using original data, (3) provided sufficient data to calculate the odds ratio (OR) with confidence interval (CI) and P-value, (4) were case-control association studies investigating **CNP** polymorphisms, (5) described the relevant genotyping primers, machines and protocols or provided reference to them, (6) diagnosed schizophrenic patients according to DSM-IV criteria, (7) used healthy individuals as controls. We searched PubMed citations (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) up to January 2009 using keywords "CNP" and
"schizophrenia".
The principle of the association is simply to determine if there is a statistically significant difference in frequency of one or more genetic markers between the case and controls. In the current study, we detect no statistical difference between the patients and controls in the respect of genotype, allele and haplotype. But we could not rule out all the possibility of this gene could play important role in the etiology of disease. Association results are the clues and hints to find the candidate region or genes; follow-up functional study should be conducted if we want to clarify the mechanisms of disease.

4) The relationship between PBL and postmortem brain $CNP$ mRNA levels is unclear, and not carefully discussed. Obviously PBLs are not myelinated, so what is the relevance of $CNP$ mRNA measurements to brain disease?

**Answer:** Most studies of gene expression in schizophrenia have focused on postmortem brain tissue; for us, it is so hard to get the tissue. However, gene expression alterations in PBLs have been used to differentiate individuals with schizophrenia from healthy controls and discordant siblings. (Middleton, F.A., et al., 2005, Tsuang, M.T., et al., 2005, Vawter, M.P., et al., 2004.) Combining gene expression profiles from easily accessible PBLs and phenotypic data may be a first step towards the biological classification of schizophrenia subtypes. Genes with altered expression in PBLs from schizophrenia previously reported to be expressed in brain (Bowden NA, et al., 2006). Consistent with previous reports from postmortem brain tissue studies in schizophrenia, changes were found in the expression of genes associated with myelination and oligodendrocyte function (Bowden NA, et al., 2006; Hakak et al., 2001; Tkachev et al., 2003). Although some of genes related to myelination were observed altered expression in PBLs from individuals with schizophrenia, whether these gene changes observed in PBLs have consequences for the pathogenesis of schizophrenia remains to be determined. In our study, we focused on $CNP$ gene, which is associated with myelination. We would like to find out if this gene mRNA expression levels were altered in the PBLs.

Following are the references:

5) The meta-analysis does not consider publication bias (e.g. funnel plot).

**Answer:** I consider the publication bias. In the Methods pg. 7, "We assessed publication bias using an ancillary procedure for funnel plot asymmetry, described by Egger et al." In the Results pg3, "No
publication bias was found." We had drawn the funnel plot but there was not bias shown. A $\chi^2$-based Q statistical test was also conducted to assess heterogeneity.

6) Given that the small number of previous genetic association reports of $CNP$ and schizophrenia are negative in East Asian sample populations, what is the rationale for this study?

**Answer:** Yes, as you stated, the association results in the Japanese and Beijing Chinese populations are negative. Previous reports showed the reduced expression level of $CNP$ in CEU populations. But no publication has investigated expression level of $CNP$ in East Asian sample populations as well as the effect of schizophrenic subtypes and drug-treatment on $CNP$ expression. In this respect, we would like to $CNP$ expression in Han Chinese and other factors like the antipsychotic’ effect on the expression levels. Also, in the meta-analysis, we tried to combine the previous association data to get a more powerful conclusion for the first time. Thus, the rationale underpinning our study was that if it is true that altered $CNP$ expression influences schizophrenia susceptibility, any direct influences on $CNP$ expression resulting from a polymorphism at the $CNP$ locus will have a similar effect.