Author's response to reviews

Title: ADHD and Disruptive behavior scores are associated with MAO-A and 5-HTT genes and with platelet MAO-B activity in adolescents

Authors:

Kerstin Malmberg (kerstin.malmberg@ki.se)
Hanna-Linn Wargelius (hanna-linn.wargelius@neuro.uu.se)
Paul Lichtenstein (paul.lichtenstein@ki.se)
Lars Oreland (lars.oreland@neuro.uu.se)
Jan-Olov Larsson (jan-olov.larsson@ki.se)

Version: 3 Date: 31 August 2007

Author's response to reviews: see over
RE: MS: 4344947721441078 - ADHD and Disruptive behavior scores are associated with MAO-A and 5-HTT genes and with platelet MAO-B activity in adolescents

Dear Editor,

On behalf of the co-authors and ourselves, we hereby resubmit our paper “ADHD and Disruptive behavior scores are associated with MAO-A and 5-HTT genes and with platelet MAO-B activity in adolescents”. We are grateful for the valuable comments by the reviewers and hope that the revision provides an adequate response to the comments. Below we detail point-by-point how we have dealt with the issues raised by the reviewers. We hope that we have satisfactorily dealt with all the comments and feel confident that the MS has been improved.

Yours Sincerely,

Dr. Kerstin Malmberg and Hanna-Linn Wargelius
We thank the reviewers for valuable and constructive comments. We hope that we have adequately dealt with all the comments and feel confident that the MS has been improved.

Reviewer 1

1) Background: Besides DRD4 and DRD5 especially the DAT gene is one of the most frequently replicated molecular correlates of ADHD!

At present polymorphisms in three dopaminergic loci stand out as the most frequently replicated molecular correlates of ADHD: DRD4, DRD5 and DAT. This information has been added in the Background section page 4 and a new reference has been added (Faraone and Khan, 2006).

2) Abstract: How is it possible to assess homozygosity in boys for the MAO-A VNTR when the polymorphism is located on a X-chromosome?

This mistake has been corrected and changed to “hemizygosity” as it is written in other parts of the manuscript.

3) It is not clear why the data are analyzed on a parametric as well as on a non-parametric level. If possible, a parametric approach must be preferred because it has always more power and is based on the exact data.

Actually, we have not used any non-parametric statistical methods in the manuscript. We guess that the reviewer refers to our use of both a dimensional and categorical classification of the behaviour problems. We would like to include both classifications because there are advantages and disadvantages with both methods (ref 6 and 7). “The probability of a disorder provides a budge between the dichotomizing nosology and the need for dimensional scales that can describe the spectrum of the disorder that is observed in the population” (ref 7).

4) The authors write that smoking affects MAO activity. Why not verifying the participants’ self report by taking cotinine levels? Smoking before taking the blood sample must be avoided and can not be controlled statistically by entering the time of the last cigarette as a covariate.

Measuring nicotine metabolite levels would have been a nice way to verify whether the subjects had been smoking within the last 48 hours. However, MAO-B activity per se does not seem to be affected by occasional smoking. Thus, Snell et al., Alc Clin Exp Res., 26, 2002, reported that in order to significantly reduce the platelet MAO-B activity, a cigarette consumption of >300 cigarettes/month is needed, which was far from the case in any of the subjects in the present study. This was further supported by the absence of any difference in MAO-B between those admitting smoking the day before and those denying.

A sentence regarding this remark has been added to the methods (p 8), to the results (p 11) and to the discussion (p 14).

5) Statistics: The rationale for the data analysis is not clear. Why analyzing boys and girls separately when a combined two-factorial MANOVA design with the additional factor sex is possible. This strategy would yield gene x sex interactions!!! The distributional variables showed skewed distributions. Why not normalizing the data before entering them into an
MANOVA model: This strategy would allow using the 5-HTTLPR as a single factor with three categories and the complex and strange method of using the LL-genotype as a reference variable (being omitted) in a GLM model could be avoided.

This information has been added to the statistical analyses (p. 10).

GLM or MANOVA could both be used when analyzing the present data and we have chosen GLM. We preferred this method especially when analyzing the dichotomized behaviour variables.

GLM could also give information about interactions. We have now analyzed interactions and added this to the methods (p 10) and to the results section (pp 11 and 12). For the dimensional ADHD/disruptive behaviour scales interactions between sex and polymorphisms in the 5HTT genotypes were analyzed using GLM. In the same way interactions between sex and platelet MAO-B activity were studied.

Instead of normalizing data (eg log-transform the dimensional behaviour variables), we preferred to apply the bootstrap method available in Stata.

Using index-variables in GLM is very similar as performing the necessary post hoc comparisons between the three genotypes in MANOVA. The omission of one index variable (“strange”) could be regarded as a standard procedure and in this paper omitting LL is in accordance with the hypothesis that LL is the low risk for the studied behaviour problems.

6) One of the major problems of the paper is that the authors do not control for zygotisity. Only one participant of each twin pair can be considered or a combination of a quantitative genetic approach with a qualitative approach must be applied.

We agree that including both twins in each pair violates the assumption in many regression models, including GLM, that the individuals in the sample should be independent. Therefore, we have used a method available in Stata that adjusts (increases) the estimated standard errors giving robust estimates of for example p-values. The method is based on the sandwich or Huber/White variance estimator a method available in Stata 9.0. Exactly this method has been used by other researchers in the area - for example in the paper recommended further down by reviewer 2 (Kim-Cohen, Caspi,, Taylor, Williams, Newcombe, Craig, et al. (2006)). This is now further described in the section about statistical analyses (p. 9).

The recommendation from the referee that the analysis is undertaken on two samples; one twin from each pair then replicated in the other sample of co-twins introduces at least two problems. The first is that the sample size will be half the available size. The statistical power would be substantially reduced. The second problem is how to interpret and present results from the two samples especially if these show to be somewhat different. We would, therefore, rather not change the sample size according to the referee but if this is regarded as important and as an improvement of the manuscript we will revise the statistical analyses.

7) Recently, it has been proposed that 5-HTT expression is not only affected by the common S/L variant of 5-HTTLPR but also by an A to G substitution (rs25531; Hu et al., 2006). Why not including this SNP into the analyses?
We are aware of the newly reported SNP within the 5-HTTLPR, however, the frequency of this SNP (the Lg variant) in the normal population is very low (ratio in caucasians is S:La:Lg 4:5:1), hence we believe it is not probable to have any major effect on the results reported here. Leaving out this SNP might be a limitation of the study, although some authors have claimed that reclassification of subjects using the SNP render comparable results to the well-established method used here and by others (see Cervilla et al., *Molecular Psychiatry* (2007) 12).

Further, the functional importance of a new classification of the 5HTTLPR is not clear, e.g. binding potential in brain does not differ between the alleles of the new classification (Parsey et al., 2006).

However, we appreciate this remark from the reviewer and we have now included this issue in the discussion (p 15).

8) The cell frequencies for the analyses are extremely small. Often n < 5!!!

We agree that the cell frequencies for the threshold diagnoses are small. However, in the analyses we have used the categorization “high dimensions of the phenotype” (sometimes called sub-threshold diagnoses). Overall these cell frequencies are not extremely small, except for the smallest 9/174 (table 1).

9) Ethnical stratification bias: What does “only a few individuals were of non-Caucasian origin” mean.

We have added more exact information concerning ethnical factors and study group (p 5) and to the discussion (p 14). Four pairs of twins (8 individuals) were of non-Caucasian origin.

10) Why not including the parent into the analyses. The authors write that such data are available?

Information about the parents is not available. The procedure during the interview with KSADS-PL is that the interview is first performed with the child alone and after that with at least one parent. The clinical interviewer then summarizes the information from both one parent (about the child) and the information from the child and classifies the symptoms as “not present” (0), “possible” (1) or “certain” (2).

This information is changed in a sentence on page 6

11) Why not correcting for multiple testing? Some of the significant results would not be stable after Bonferroni-correction.

The Bonferroni corrections is very often discussed (eg Perneger (1998) and statisticians seem to have rather different opinions regarding the use of adjustment methods for multiple comparisons. We agree that some of the results would not be stable after a Bonferroni adjustment. This is a very conservative method and one problem with Bonferroni adjustment is that the type II errors are increased so that truly important differences can be regarded as non-significant. The multiple comparisons problem could be dealt with by describing what tests of significance have been performed and we have tried to comment on this in the text (p 9). We would prefer not to use adjustment for multiple comparisons, for example Bonferroni in this paper.
Reviewer 2

1. The authors undertake a candidate gene association study for ADHD, ODD and CD defined categorically and dimensionally, using a twin sample. Analysis is undertaken on pairs and then S.E. corrected. However MZ twin pairs are genetically the same, why include both? It does not seem sensible to have analysed the data in this way. It is recommended that the analysis is undertaken on a sample composed of one twin from each pair then replicated in the other sample of co-twins.

Please see answer to reviewer 1, comment 6.

2. Page 5 Blood samples were obtained from 247 individuals-from how many pairs?

Blood samples were obtained from 247 individuals (123 twin pairs and one individual), 106 boys and 141 girls. This information has been added to page 7.

3. How were parent and child reports from interview integrated for each type of psychopathology?

The procedure during the interview with KSADS-PL was that the interview was first performed with the child alone and after that with at least one parent. The clinical interviewer (the first author, KM) then summarized the information from both the parent (about the child) and the information from the child and classifies the symptoms as “not present” (0), “possible” (1) or “certain” (2).

The information about the procedure is now revised (p. 6)

4. Page 10 The gender ratio of ADHD combined type is much lower than in other studies (male: female 7,8:1 in non referred, 3,4:1 in referred populations). Can the authors explain this? Was it attributable to the way information was integrated across informants?

Meta-analyses of childhood ADHD have shown male:female ratio of 3:1 in non referred populations eg Gaub et al 1997. We found 1.6:1 in our study for subthreshold diagnosis ADHD combined type. Studies of adult ADHD have shown ratios of 1.6:1 (Kessler et al 2006) and even 1:1 (Faraone and Biedermann 2005). The ratio in the present study could be rather low because our sample consists of adolescents (average age 16 yrs) that are between childhood and adulthood whereas in other studies subjects are usually children or adults. In another study from our own group there were no differences in Hyperactivity/Impulsivity scale and rather small but significant differences in Inattention at age 16-17 (Larsson et al 2006, JAACAP).

This information has been added to the discussion (p 13).

5. Page 11-MAOA VNTR 66% of the subjects. “Hemizygous”. Do the authors mean homozygous?

We accidentally wrote homozygous in the abstract which might have been confusing, this is now changed to hemizygous. Since the MAOA VNTR is located on the X-chromosome, boys will have only one copy of the allele and hence cannot be homozygous.
6. Page 13 Confounders for MAOA B activity. Could there be other confounders additional to smoking?

MAO B activity differs significantly between the sexes, however, we have analyzed boys and girls separately. Besides that, the use of MAO B inhibitors would of course affect the activity, but none of the subjects had such treatment. Except for Lithium and some old anti-depressives, no other compounds seem to be known to affect MAO B activity. None of those were used of any of the subjects.

7. Conclusions. The authors need to highlight findings of meta-analyses as a number have now been published on ADHD and these variants. This provides the reader with a context to the findings from this paper. Are they likely to be false positive, false negative? How do they compare with findings from meta-analyses? What does their own study add to previous findings? For example, Faraone et al, 2005 on ADHD and MAOA and 5HTT variants Kim-Cohen et al, 2006-meta-analysis showing no main effect of MAOA variant on antisocial behaviour

We have extended the discussion with regard to especially MAOA (p 14-15). We have also tried to emphasize the inconsistencies of different studies in this field, and also to draw some attention to the limitations of this study (p15).