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ARTICLE

Epistatic interaction between the monoamine oxidase A and serotonin transporter genes in anorexia nervosa

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The serotonin (5-HT) and norepinephrine (NE) systems are likely involved in the aetiology of anorexia nervosa (AN) as sufferers are premorbidly anxious. Specifically, we hypothesize that genes encoding proteins, which clear 5-HT and NE from the synapse, are prime candidates for affecting susceptibility to AN. Supporting our hypothesis, we earlier showed that the NE transporter (NET) and monoamine oxidase A (MAOA) genes appear to contribute additively to increased risk of developing restricting AN (AN-R). With regard to the *MAOA* gene, a sequence variant that increases MAOA activity and has suggested association with the anxiety condition, panic disorder was preferentially transmitted from parents to affected children. Here we provide evidence in support of interaction between the *MAOA* and serotonin transporter (SERT) genes in 114 AN nuclear families (patient with AN plus biological parents). A *SERT* gene genotype with no apparent individual effect on risk and known to be associated with anxiety is preferentially transmitted to children with AN (χ^2 trend = 9.457, 1 df, $P = 0.0021$) and AN-R alone (χ^2 trend = 7.477, 1 df, $P = 0.0063$) when the 'more active' *MAOA* gene variant is also transmitted. The increased risk of developing the disorder is up to eight times greater than the risk imposed by the *MAOA* gene variant alone – an example of synergistic epistatic interaction. If independently replicated, our findings to date suggest that we may have identified three genes affecting susceptibility to AN, particularly AN-R: the *MAOA*, *SERT*, and *NET* genes.
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Introduction

Individuals suffering from anorexia nervosa (AN) perceive themselves as fat even when emaciated, and pursue thinness through food restriction (AN-R) or purging (AN-BP). 'Anxiety' genes likely contribute to the suggested genetic component in AN as sufferers are usually anxious prior to developing the disorder.[1,2] The 'anxiety' neurotransmitters serotonin (5-HT) and norepinephrine (NE) are

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cleared from the synaptic cleft by the 5-HT transporter (SERT/SLC6A4), the NE transporter (NET/SLC6A2), and monoamine oxidase A (MAOA) proteins.[3] The genes that encode these proteins are consequently prime candidates for involvement in AN aetiology. The *MAOA* gene promoter contains a functional polymorphism known as the *MAOA*-uVNTR that has long (*MAOA*-L) and short (*MAOA*-S) variant forms due to a repeated 30-bp sequence. *MAOA*-L (4 repeats or rarely 3 repeats plus the first 18-bp of the 30-bp sequence) is transcribed more efficiently than *MAOA*-S (3 repeats of the 30-bp sequence). The transcriptional efficiency of another two rare alleles, 2 or 5 repeats, is unknown or debatable, respectively.[4,5] In male skin fibroblast cultures, the *MAOA*-L 4-repeat allele results in increased MAOA activity relative to *MAOA*-S.[6] *MAOA*-L has suggestive association with panic disorder in females.[5] The *SERT* gene promoter contains a functional polymorphism known as the 5-HTTLPR due to a 44-bp insertion (5-HTTLPR-L) or deletion (5-HTTLPR-S). The transcriptional activity of 5-HTTLPR-S is less than half that of 5-HTTLPR-L. Genotypes with one or two copies of 5-HTTLPR-S halve SERT uptake of 5-HT relative to the 5-HTTLPR-L/L genotype and appear associated with anxiety-related traits.[7]

In an earlier study, we reported association of AN-R with the long form of a polymorphism in a repetitive sequence known as the NETpPR in the *NET* gene promoter, the only published association study with this polymorphism to date.[8] By itself (independently) the long form of the NETpPR polymorphism more than doubled risk for AN-R. Shortly after in a family-based study (ie parents and their children), we reported that *MAOA*-L is preferentially transmitted to children with AN-R if the long form of the NETpPR polymorphism is also transmitted.[9] The contribution by *MAO*-L and the long form of the NETpPR polymorphism to increased risk of developing AN-R appeared to be additive, that is, the sum of their independent contributions, although the independent risk contributed by *MAOA*-L did not reach statistical significance in case control (using control data from the literature) or family-based analyses. The independent contribution by *MAOA*-L to increased risk of panic disorder is also thought to be small.[5] To date no one has attempted a replication study to confirm or refute our findings regarding *MAOA*-L.[9] Recently, we found no evidence for an independent contribution by the 5-HTTLPR to AN susceptibility in our family-based sample of at least a genotypic relative risk of 2.0 with 93% statistical power (odds ratio 1.0, 95% CI 0.7–1.5),[10] which confirmed independent findings by others in three earlier studies.[11–13] Data from the only other independent study of AN and the 5-HTTLPR suggested association of AN-BP with the 5-HTTLPR-S allele and 5-HTTLPR-S/S genotype (both $P=0.02$).[14] Additionally, we found no suggestion of interaction between the NETpPR polymorphism and the 5-HTTLPR, that is, neither 5-HTTLPR allele appeared to increase risk of AN when 5-HTTLPR allele transmissions from 5-HTTLPR-S/L heterozygous parents were stratified based on the NETpPR genotypes of their children with AN.[10] To date no one has attempted a replication study to confirm or refute our negative interaction findings between the NETpPR polymorphism and the 5-HTTLPR.[10] In the current study, to further investigate our hypothesis that proteins which clear NE and 5-HT from the synaptic cleft modify susceptibility to develop AN, we assess potential interaction between the *MAOA*-uVNTR and 5-HTTLPR in 114 nuclear families. Findings from our analysis suggest that increased risk of AN due to the combined effects of these polymorphisms is greater than additive, meaning that these functional polymorphisms are interacting epistatically.

Materials and methods

Subjects

We recruited 114 consecutive consenting unrelated patients with AN (110 Caucasian:4 East Asian) and their parents (106 trios, eight duos) in Australia. Each trio consisted of one DSM-IV[15] patient with AN plus both biological parents. Each duo consisted of one DSM-IV female patient with AN plus her biological mother. The trios and duos participated in our earlier studies in which they are described in detail.[8–10] Briefly, the patients with AN consisted of 109 females (aged 16.8 ± 3.5 years at assessment, onset of AN 2.0 ± 2.0 years before assessment, minimum BMI measured in hospital after onset of AN: 14.6 ± 1.7) and five males (aged 16.5 ± 3.8 years, onset of AN 1.6 ± 1.0 years before assessment, minimum BMI measured in hospital after onset: 14.7 ± 1.2). Senior psychiatrists confirmed the diagnosis of DSM-IV AN blind to genotype using a structured clinical interview plus medical record data. We gained ethics approval from the appropriate institutional Committees on Human Experimentation in accord with the Helsinki Declaration of 1975. All participants gave written informed consent but parents signed for those aged under 14 years.

Molecular genetic methods

The PCR-based genotyping methods for detecting the *MAOA*-uVNTR and 5-HTTLPR alleles are described in our earlier reports.[9,10] The *MAOA*-uVNTR genotypes of the 114 patients with AN consisted of 11 *MAOA*-S/S, 43 *MAOA*-S/L, 55 *MAOA*-L/L, and five *MAOA*-L males. The *MAOA*-uVNTR genotypes of their mothers consisted of 16 *MAOA*-S/S, 55 *MAOA*-S/L, and 43 *MAOA*-L/L. The fathers' *MAOA*-uVNTR genotyping data could not be analysed (and are therefore not listed) as males have only one allele for X chromosome genes, for example the *MAOA* gene[16] and therefore preferential transmission of alleles cannot be estimated. The father's *MAOA*-uVNTR genotype was used only to determine which allele the mother transmitted in the trios. We could deduce the mother's transmitted *MAOA*-uVNTR

allele in all duos without knowledge of the father's genotype as no duo was composed of two *MAOA*-S/L heterozygotes.

5-HTTLPR genotyping data were available for the mother, father and their child (AN patient) in each of the 106 trios, and for the mother and her daughter (AN patient) in each of the eight duos. The 5-HTTLPR genotypes of the 114 patients with AN consisted of 24 5-HTTLPR-S/S (22 of these from the trios), 59 5-HTTLPR-S/L (57 of these from the trios), and 31 5-HTTLPR-L/L (27 of these from the trios). The 5-HTTLPR genotypes of the 114 mothers consisted of 29 5-HTTLPR-S/S (all from the trios), 51 5-HTTLPR-S/L (46 of these from the trios), and 34 5-HTTLPR-L/L (31 of these from the trios). The 5-HTTLPR genotypes of the 106 fathers in the trios consisted of 22 5-HTTLPR-S/S, 55 5-HTTLPR-S/L, and 29 5-HTTLPR-L/L. *All* and *only* transmissions from the 101 5-HTTLPR-S/L parents (46 mothers, 55 fathers) in the trios were included in Table 2 'Trio parents' analysis. In Table 2 'Trio and duo mothers' analysis, it was necessary to exclude 12 'trio' mothers and 1 'duo' mother as all mothers, fathers, and children in these trios, and the mother and daughter in the duo, consisted of 5-HTTLPR-S/L heterozygotes and so it was not possible to determine which allele was transmitted by the mother. Therefore *all* and *only* transmissions from 38 5-HTTLPR-S/L mothers were included in Table 2 'Trio and duo mothers' analysis (ie 51 total 5-HTTLPR-S/L mothers *minus* 1 5-HTTLPR-S/L 'duo' mother *minus* 12 5-HTTLPR-S/L 'trio' mothers = 38 mothers).

Statistical analysis

Logistic regression is useful for detecting epistatic interactions involving at least *two* independent variables when the distribution of responses on the dependent variable is expected to be nonlinear for at least one independent variable, although calculations become problematic when genotype combinations result in few data points. Novel methods employing mainly case control data are being developed.[17,18] We used the Armitage–Cochrane trend test to analyse Table 1 data because (a) there were two variables in our analyses including *one* ordinal independent variable (ie genotype) and a binomial dependent variable (ie allele transmitted), the binomial proportions across genotypes were linear, and we were posing the question 'Is there a consistent trend towards decrease or increase in the difference in transmission of *MAOA*-L and *MAOA*-S alleles between genotype groups?' The Armitage–Cochrane trend test is a statistically powerful test with 1 degree of freedom, which may be seen as both an advantage and disadvantage, the latter due to greater likelihood of a type 1 error. Consequently we supported our analysis of Table 1 with the Fisher–Freeman–Halton exact test for $R \times C$ contingency tables, a generalized version of the conservative Fisher's exact test. We used only the Fisher–Freeman–Halton exact test for Table 2 data analysis as binomial proportions across genotypes were not linear. We used *SPSS Sample Power* for $K \times C$ Tables to determine statistical power (two-tailed). We used two-sided *P*-values and a 0.05 level of significance.

We used the following method to test the hypothesis of epistatic interaction being present: If no trend was detected (ie $P \geq 0.05$) then no evidence of epistasis was suggested. If a trend was detected ($P < 0.05$) epistatic interaction was suggested as the 5-HTTLPR does not appear to contribute independently to risk of AN[10–13] and hence the trend could not be due to an additive effect. Synergistic or antagonistic epistatic interaction was suggested if the degree of risk was increased above or decreased below the risk of AN associated with *MAOA*-L alone, respectively.

In regard to simultaneous testing, the only analyses previously conducted on the sample in this report are those described in our studies involving three biallelic

Table 1 Epistatic interaction analysis: mothers' *MAOA*-uVNTR TDT data stratified by 5-HTTLPR genotypes of their AN children

	Allele transmitted from MAOA-S/L mother to AN child (AN-R only)		
AN child's 5-HTTLPR genotype	*MAOA-L*	*MAOA-S*	*MAOA-L odds ratio (95% CI) for all AN*
5-HTTLPR-S/S	11 (9)	1 (1)	11.0 (1.6–474)
5-HTTLPR-S/L	17 (16)	13 (11)	1.3 (0.6–2.9)
5-HTTLPR-L/L	4 (3)	9 (7)	0.4 (0.1–1.6)
Combined	32 (28)	23 (19)	1.4 (0.8–2.5)

Table 2 Epistatic interaction analysis: parents' 5-HTTLPR TDT data stratified by *MAOA*-uVNTR genotypes of their AN children

	Allele transmitted from 5-HTTLPR-S/L parent to AN child (AN-R only)		
AN child's MAOA-uVNTR genotype	*5-HTTLPR-S*	*5-HTTLPR-L*	*5-HTTLPR-S odds ratio (95% CI) for all AN*
Trio and duo mothers			
MAOA-L/L or *MAOA*-L[a]	7 (7)	9 (7)	0.8 (0.2–2.3)
MAOA-S/L	11 (9)	8 (7)	1.4 (0.5–3.9)
MAOA-S/S	1 (1)	2 (2)	0.5 (0.01–9.6)
Combined	19 (17)	19 (16)	1.0 (0.5–2.0)
Trio parents			
MAOA-L/L or *MAOA*-L[b]	24 (23)	27 (22)	0.9 (0.5–1.6)
MAOA-S/L	24 (17)	18 (17)	1.3 (0.7–2.6)
MAOA-S/S	2 (1)	6 (6)	0.3 (0.03–1.9)
Combined	50 (41)	51 (45)	1.0 (0.7–1.5)

[a]Stratum includes one male; [b]stratum includes four males.

polymorphisms and two interactions between them.[8–10] The current report analyses the third interaction.

Results

We tested the hypothesis that the *MAOA*-uVNTR and 5-HTTLPR interact epistatically in AN. We combined *MAOA*-uVNTR and 5-HTTLPR genotyping data from our earlier studies[9,10] with 5-HTTLPR genotyping data of the eight duos and *MAOA*-uVNTR genotyping data of 19 trios determined in the current study leading to an increase in *MAOA*-uVNTR and 5-HTTLPR genotyping data of 20 and 8%, respectively, over that reported in our earlier studies.[9,10] However, the odds ratio of 1.4 for *MAOA*-L (combined sample in Table 1) and odds ratio of 1.0 for 5-HTTLPR-S (combined sample in Table 2) remained the same as we reported earlier.[9,10] *MAOA*-uVNTR genotypes of patients and their mothers, and 5-HTTLPR genotypes of patients and parents were in Hardy–Weinberg equilibrium (data analysis not shown).

We tested for epistatic interaction between the 5-HTTLPR and *MAOA*-uVNTR through contingency table analysis by (a) stratifying allele transmissions from *MAOA*-S/L heterozygous mothers, that is, transmission disequilibrium test data[19] based on the 5-HTTLPR genotypes of their affected children (Table 1), and (b) stratifying allele transmissions from 5-HTTLPR-S/L mothers and trio parents, that is, transmission disequilibrium test data[19] based on the *MAOA*-uVNTR genotypes of their affected children (Table 2). Epistatic interaction is suggested from the data in Table 1 (Armitage–Cochrane χ^2 trend test = 9.457, 1 df, $P = 0.0021$; Fisher–Freeman–Halton exact test $P = 0.0061$). The power of the χ^2 test of independence of the data in the contingency table is 80%. *MAOA*-L is preferentially transmitted from mothers to affected children having the 5-HTTLPR-S/S genotype (odds ratio: 11.0, 95% CI 1.6–474) but not to affected children having the 5-HTTLPR-L/L genotype (odds ratio: 0.4, 95% CI 0.1–1.6). AN-R data alone ($n = 98$ nuclear families) produce a similar finding (Armitage–Cochrane χ^2 trend test = 7.477, 1 df, $P = 0.0063$). However, the reciprocal analysis in Table 2 provides no evidence of epistatic interaction as neither 5-HTTLPR allele is preferentially transmitted when conditioned on the *MAOA*-uVNTR genotype of the affected child: Fisher–Freeman–Halton exact test $P = 0.238$ for trio parents' data and $P = 0.619$ for mothers' data alone in the entire AN sample. In support of our finding in Table 1, *MAOA*-uVNTR and 5-HTTLPR genotypes are independently distributed (Table 3) in mothers and AN daughters (Fisher–Freeman–Halton exact test $P = 0.271$ and $P = 0.734$, respectively) suggesting that observed nonrandom transmissions of *MAOA*-uVNTR alleles from mothers to daughters according to 5-HTTLPR genotypes of the latter do not just reflect a nonrandom distribution of 5-HTTLPR genotypes in our sample, independent of disease status.

Discussion

Risk of AN appears to increase in a 5-HTTLPR-S/S individual who also receives a *MAOA*-L allele from an *MAOA*-S/L mother and may be eight times greater (odds ratio = 11) than the risk imposed by *MAOA*-L alone (odds ratio = 1.4) implying synergistic epistatic interaction between the *MAOA*-uVNTR and 5-HTTLPR. Epistatic interaction has not previously been observed between the *MAOA*-uVNTR and the 5-HTTLPR and cannot be due to proximity as these polymorphisms are on different chromosomes.[16,20] However, other functional sequence variation in linkage disequilibrium with either polymorphism may be contributing to AN susceptibility. In contrast, risk of AN appears *not* to increase in a *MAOA*-L/L individual who also receives a 5-HTTLPR-S allele from a 5-HTTLPR-S/L mother. We suggest two explanations for the discrepancy in the reciprocal analyses. Firstly, epistatic interaction suggested by Table 1 data may be a false positive. We measured preferential transmission of *MAOA*-uVNTR alleles from only mothers as the *MAOA* gene is on the X chromosome and consequently our sample size was restricted. Secondly, if there are two 5-HTTLPR-S alleles, for example, S1 and S2, the *MAOA*-uVNTR may interact with, for example, S1 (or another functional variant in linkage disequilibrium with S1) but not with S2. Preferential transmission of *MAOA*-L to 5-HTTLPR-S/S children is then due to 5-HTTLPR-S/S being 5-HTTLPR-S1/S1. In the reciprocal analysis, the interaction is not seen due to 5-HTTLPR-S/L mothers being S1/L and S2/L. Both S1 and S2 will be transmitted but if S2 is more

Table 3 Joint distribution of *MAOA*-uVNTR and 5-HTTLPR genotypes of mothers and their AN daughters

	MAOA-uVNTR genotype					
	109 AN daughters			*114 Mothers*		
5-httLPR genotype[a]	*MAOA-L/L*	*MAOA-S/L*	*MAOA-S/S*	*MAOA-L/L*	*MAOA-S/L*	*MAOA-S/S*
5-HTTLPR-S/S	12	10	2	11	14	4
5-HTTLPR-S/L	28	23	4	15	26	10
5-HTTLPR-L/L	15	10	5	17	15	2

[a]Five males removed as their *MAOA*-uVNTR genotype consists of one allele.

common than S1 the epistatic interaction between the *MAOA*-uVNTR and 5-HTTLPR-S1/S1 suggested in Table 1 will be masked. If our findings in Tables 1 and 2 are independently replicated, a search should be conducted for 5-HTTLPR-S variants (including sequence variation in linkage disequilibrium with 5-HTTLPR-S) as three rare 5-HTTLPR-S variant alleles have been observed.[21] A 5-HTTLPR-S variant may also form a haplotype with an allele of the intron 2 functional polymorphism[22] (or another functional sequence variant nearby), which epistatically interacts with *MAOA*-L.

Until independently confirmed we cautiously interpret our earlier[8–10] and current findings, but suggest that we may have identified three genes involved in susceptibility to AN, particularly AN-R: the *MAOA*, *SERT*, and *NET* genes, with the *MAOA* gene serving as the 'hinge-pin' as we have provided evidence in support of preferential transmission of *MAOA*-L in separate analyses with the *NET* and *SERT* genes but detected no interaction between the *NET* and *SERT* genes (at least involving the *NET* gene NETpPR polymorphism and *SERT* gene 5-HTTLPR). We could not globally assess interaction as the *MAOA*-uVNTR restricted our sample size (ie only maternal *MAOA*-uVNTR allele transmissions were informative) resulting in empty cells. Neither could we globally assess joint genotype distributions due to low frequency of genotypes homozygous for the short form of the NETpPR polymorphism which again resulted in empty cells. However, pairwise comparisons (Table 3, and data not shown) provide evidence to support independent distribution of NETpPR, *MAOA*-uVNTR, and 5-HTTLPR genotypes (all Fisher–Freeman–Halton exact test P-values >0.120 for parents and children), which suggests that nonrandom transmission of alleles in our sample does not reflect nonrandom distribution of genotypes independent of disease status.

Our findings may help elucidate abnormalities in long-term weight-recovered AN individuals whose biology likely approximates that of the premorbid state before starvation confounds observations. Preferential transmission of *MAOA*-L with consequent increased activity of MAOA can explain elevated CSF 5-hydroxyindoleacetic acid (5-HIAA) levels in recovered individuals[23] as MAOA catalyses the breakdown of 5-HT to 5-HIAA. Preferential transmission of 5-HTTLPR-S/S can explain reduced binding of 5-HT at the 5-HT2a receptor in the mesial temporal cortex of persons who have recovered from AN-R.[24] 5-HTTLPR-S/S leads to reduced expression and reuptake of 5-HT by the SERT so that intrasynaptic 5-HT increases.[7] 5-HT2a receptors should downregulate in response to increased 5-HT leading to reduced binding at this receptor. Further evidence for increased intrasynaptic 5-HT is the reduced anxiety experienced by recovered AN patients after depleting the precursor of 5-HT, tryptophan.[25] As the authors rightly state,[25] tryptophan depletion decreases the blood tryptophan:tyrosine ratio thereby diminishing transport of tryptophan across the blood–brain barrier and consequently 5-HT synthesis. However, they do not state that tryptophan depletion also increases transport of tyrosine across the blood–brain barrier and enhances CSF levels of NE known to be low in individuals after recovery from AN.[26] Preferential transmission of *MAOA*-L can explain low CSF NE in AN patients after recovery as MAOA breaks down NE. Catechol-*o*-methyltransferase (COMT) also breaks down NE and could contribute to low CSF NE in patients after recovery. However, only one of two studies involving AN and the Val158Met polymorphism in the *COMT* gene showed positive association with the allele that encodes a more active COMT variant.[27] The other study found no association with either allele.[28]

Our findings may be important for treatment. As 5-HT appears high (shown by high CSF 5-HIAA) relative to NE in persons after recovery from AN, we propose that treatment be focused on balancing levels of 5-HT and NE in the brain of these individuals. If our cited and current findings are independently replicated, we plan a clinical trial in which L-tyrosine is administered to *increase* and *decrease* CSF tyrosine and tryptophan, respectively, through competition across the blood–brain barrier. CSF NE should *rise* and 5-HT should *fall* (relative to each other) due to *increased* and *decreased* synthesis, respectively. After a short 'tyrosine-free' period, drugs that increase intrasynaptic NE will be given. Additionally, we intend matching genotype with treatment response to try to predict drug efficacy in AN.

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