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Investigation of Epistasis Between the Serotonin Transporter and Norepinephrine Transporter Genes in Anorexia Nervosa

Ruth E Urwin*[1,5], Bruce H Bennetts[2,6], Bridget Wilcken[3,6], Pierre JV Beumont[4,5], Janice D Russell[5], Kenneth P Nunn[1,5]

[1]Department of Psychological Medicine, The Children's Hospital at Westmead, Westmead, NSW, Australia; [2]Department of Molecular Genetics, The Children's Hospital at Westmead, Westmead, NSW, Australia; [3]Department of Biochemical Genetics and Newborn Screening, The Children's Hospital at Westmead, Westmead, NSW, Australia; [4]Department of Psychiatry, Royal Prince Alfred Hospital, Camperdown, NSW, Australia; [5]Discipline of Psychological Medicine, University of Sydney, NSW, Australia; [6]Discipline of Paediatrics and Child Health, University of Sydney, NSW, Australia

Weight-restored patients with anorexia nervosa (AN) respond favorably to the selective serotonin reuptake inhibitor fluoxetine, which justifies association studies of the serotonin transporter gene (*SLC6A4*, alias *SERT*) and AN. Case–control studies suggest that the least transcriptionally active allele of the *SERT* gene promoter polymorphism (5-HTTLPR) has an increased frequency in AN patients. However, this finding was not replicated with 55 trios (AN child + parents) and the transmission disequilibrium test (TDT). To clarify the role of the 5-HTTLPR in susceptibility to AN, we used the TDT and 106 Australian trios to provide 93% power to detect a genotypic relative risk (GRR) of 2.0. Our results were negative for this GRR (McNemar's $\chi^2 = 0.01$, df = 1, $p = 0.921$, odds ratio 1.0, 95% CI 0.7−1.5). Additionally, we found no association with AN females, AN subtype, age at onset, or minimum BMI. We then performed the first reported investigation of epistasis between the *SERT* gene and norepinephrine transporter gene (*SLC6A2*, alias *NET*) in AN, as an earlier study suggested that atypical AN responds to the dual serotonin–norepinephrine reuptake inhibitor venlafaxine. We observed no epistasis between the 5-HTTLPR and a polymorphism within the *NET* gene promoter polymorphic region (NETpPR) ($\chi^2 = 0.48$, df = 1, $p = 0.490$). Although 5-HTTLPR modulates serotonin reuptake by the serotonin transporter, our analyses provide no evidence that susceptibility to AN is modified by 5-HTTLPR alone, nor in concert with as yet undetermined functional effects of the NETpPR polymorphism.

Neuropsychopharmacology (2003) **28,** 1351–1355, advance online publication, 14 May 2003; doi:10.1038/sj.npp.1300204

Keywords: genetic epistasis; eating disorders; anorexia nervosa; norepinephrine; serotonin; carrier proteins

INTRODUCTION

DSM-IV (American Psychiatric Association, 1994) anorexia nervosa (AN) is diagnosed when body weight remains below 85% of normal, and there is an intense fear of gaining weight, a disturbed perception of one's shape and/or weight, and amenorrhea (in females). Weight is controlled by food restriction (AN-R) or purging (AN-BP). Atypical AN meets all AN-R or AN-BP diagnostic criteria except that weight remains just above the diagnostic threshold or amenorrhea is absent. Serotonergic dysfunction is implicated in AN pathophysiology (Brewerton and Jimerson, 1996) as long-term, weight-restored patients have elevated CSF levels of the serotonin (5-HT) metabolite 5-hydroxyindoleacetic acid (Kaye *et al*, 1991a). Additionally, the selective 5-HT reuptake inhibitor (SSRI) fluoxetine targeted at the 5-HT transporter may be clinically useful in weight-restored AN patients (Kaye *et al*, 1991b; Kaye *et al*, 2001). These observations, and twin and family data suggesting a genetic component in AN (Holland *et al*, 1988; Strober *et al*, 2000), justify association studies of AN and the 5-HT transporter gene (*SLC6A4*, alias *SERT*). Within the *SERT* gene promoter lies the 5-HT transporter-linked polymorphic region (5-HTTLPR). 5-HTTLPR is a functional polymorphism with two common alleles. The short (*S*) allele consists of 14 repeats of a 20−23-bp sequence. The long (*L*) allele consists of 16 repeats having transcriptional activity triple that of the *S* allele (Heils *et al*, 1996). The *L/L* genotype doubles 5-HT transporter expression relative to genotypes carrying one or two *S* alleles (Lesch *et al*, 1996) justifying two genotype groups (*L/L* vs *S/S* + *S/L*) for association studies. Rare variants include a 15-repeat allele (Nakamura *et al*, 2000)

with undetermined effect on transcription. Case–control studies (Di Bella *et al*, 2000; Sundaramurthy *et al*, 2000; Fumeron *et al*, 2001) demonstrate a trend towards increased frequency of the *S* allele, and *S/S* or *S/L* genotypes in AN patients, reaching statistical significance for AN-BP patients once (Di Bella *et al*, 2000) ($p = 0.020$, odds ratio 1.9, 95% CI 1.1–3.1). However, the one family-based study (Hinney *et al*, 1997), which used the transmission disequilibrium test (TDT) (Spielman *et al*, 1993) and 55 trios (AN child + parents), provided no evidence for preferential transmission of the *S* allele as 52 *S/L* parents transmitted 27 *S* and 25 *L* alleles to AN children ($p = 0.90$). Disagreement between the case–control and family-based findings may involve population stratification bias in the case–control studies. However, if 5-HTTLPR has little involvement in AN, a false negative result in the family-based study is not ruled out as it has only 67% power to detect a susceptibility allele with a genotypic relative risk (GRR) of 2.0.

Data also point to noradrenergic dysregulation in AN. Long-term, weight-restored AN patients have low norepinephrine levels in blood and CSF (Kaye *et al*, 1985; Pirke *et al*, 1992). Furthermore, venlafaxine, which blocks reuptake by 5-HT and norepinephrine transporters, may be useful for treating atypical AN (Ricca *et al*, 1999). Additionally, we recently demonstrated strong association between a polymorphism in the norepinephrine transporter gene (*SLC6A2*, alias *NET*) promoter polymorphic region (NETpPR) and AN-R (Urwin *et al*, 2002). Although a gene's independent contribution to disease may avoid detection, its involvement in disease susceptibility may become apparent through epistasis (gene–gene interaction) (Ritchie *et al*, 2001; Culverhouse *et al*, 2002). It is important to know whether susceptibility to develop AN increases when DNA sequence variations in different genes occur simultaneously. We hypothesized that there is epistasis between the *SERT* and *NET* genes in AN, and through investigating this hypothesis report the first investigation of epistasis between these genes. Additionally, to clarify whether 5-HTTLPR is independently involved in AN we employ the TDT and 106 Australian trios to provide 80 and 93% power to detect GRRs of 1.75 and 2, respectively.

MATERIALS AND METHODS

Subjects

Our 106 unrelated trios (102 Caucasian, four East Asian) were recruited from Sydney and Melbourne (Australia) and included the 101 trios reported in Urwin *et al* (2002), in which we found AN-R to be associated with the NETpPR L4 allele and L4/L4 genotype, plus five trios recruited later. Each trio consisted of one DSM-IV AN patient plus both biological parents. Families were excluded if both parents were unavailable for testing. Ethics approval had been gained from the appropriate institutional Committees on Human Experimentation in accord with the Helsinki Declaration of 1975. All participants gave written informed consent (parents signed for children aged under 14 years). Patients were consecutive consenting inpatients ($n = 98$) or outpatients ($n = 8$) admitted for treatment of AN and all currently fulfilled/had previously fulfilled DSM-IV AN criteria, except presence of amenorrhea was waived for

males and prepubertal females. Diagnosis of AN (including AN-R and AN-BP) was made blind to genotype and involved longitudinal assessment and case note searches. Diagnosis was made by eating disorder specialists, and confirmed with 95% consensus by one of three psychiatrists (KPN, JDR, PJVB) after a personal interview and examination of collated data. KPN reviewed the case notes to confirm/disconfirm the diagnosis where diagnostic ambiguity existed. Ages and BMI were recorded during assessment. BMI was determined from weight and height measured by hospital staff, the DSM-IV AN weight criterion being based on the minimum BMI recorded. At minimum BMI, all AN patients aged 11–15 years weighed less than or equal to their age and sex-specific 5th percentile BMI cutoff as determined in an Australian population study (Harvey and Althaus, 1993). Above 15 years of age a BMI of 17.5 was the upper limit (WHO, 1992). There were 101 female (87 AN-R, 14 AN-BP) and five male patients (three AN-R, two AN-BP). Of the 101 females, 28 were aged under 15 years (mean age 13.60 ± 1.25 years) at their minimum BMI (mean minimum BMI 14.20 ± 1.61) reached at a mean of 0.92 ± 0.58 years after onset of AN, and 73 were aged 15 years and over (mean age 18.18 ± 3.46 years) at minimum BMI (mean minimum BMI 14.70 ± 1.75) reached at a mean of 2.38 ± 2.24 years after onset of AN. The five males were aged 13.50, 13.70, 14.70, 18.00, and 22.40 years at minimum BMI values of 14.54, 15.08, 15.56, 12.64, and 15.43, respectively, reached 2.50, 0.60, 0.30, 2.00, and 2.40 years after onset of AN, respectively.

Molecular Genetic Methods

EDTA venous blood (10 ml) was collected from patients and parents. Genomic DNA was extracted from the blood using standard methods (Miller *et al*, 1988). 5-HTTLPR was PCR-amplified using previously published primer sequences (Heils *et al*, 1996): forward primer stpr5 5'-GGCGTTGCCGCTCTGAATGC (positions −1416 to −1397) and reverse primer stpr3 5'-GAGGGACTGAGCTGGA-CAACCAC (positions −910 to −888). Base numbering relates to the start site of the Heils *et al* (1996) sequence. Amplification conditions from Heils *et al* (1996) were modified as follows. PCR reactions were performed in 10 μl volumes containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 0.5 μM of each primer, 200 μM of each dNTP (7-deaza-dGTP replaced all dGTP), 5% dimethyl sulfoxide, 0.25 U of Ampli-*Taq* Gold (Applied Biosystems, Foster City, CA, USA), and 50 ng DNA template. The GeneAmp PCR System 9700 (Applied Biosystems) was used for thermal cycling: 95°C for 10 min; 30 cycles of 95°C for 30 s, 67–52.5°C for 30 s (decreased by 0.5°C/cycle over the 30 cycles from 67 to 52.5°C), 72°C for 40 s; 10 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 40 s; and finally 72°C for 10 min. PCR products were separated by size on 8% polyacrylamide, followed by silver staining using a modification of the Merril *et al* (1981) method (ie 5 min 10% ethanol, 3 min 3% nitric acid, two water washes, 15 min 0.1% silver nitrate, one water wash, incubation in 50 ml 3% NaOH containing 40 μl 37% formaldehyde until desired staining was achieved, two water washes, 5 min 3% acetic acid, one water wash). Direct DNA sequencing of a 16-repeat homozygote PCR product using the ABI Prism Big Dye Terminator Ready Reaction Kit

(Applied Biosystems) and the ABI PRISM 377 DNA Sequencer (Applied Biosystems) confirmed amplification of the correct DNA sequence. A known 14-repeat/16-repeat heterozygote and DNA standard were used to identify 5-HTTLPR variants.

The 343-bp NETpPR within the *NET* gene promoter region includes six AAGG repeat islands (AAGG1−AAGG6). The methodology to detect a 4-bp deletion(S4)/insertion(L4) polymorphism in AAGG4 is described in Urwin *et al* (2002), in which AN-R was associated with L4 and the L4/L4 genotype. NETpPR genotyping data for 90 AN-R trios in the current study (87 trios genotyped for Urwin *et al* (2002) plus three trios recruited and genotyped later) were (transmitted : nontransmitted) alleles L4 (145 : 123), S4 (35 : 57) and genotypes L4/L4 (58 : 41), S4/L4 (29 : 41), S4/S4 (3 : 8). (NB. nontransmitted genotype = two nontransmitted alleles). L4/L4 was preferentially transmitted to AN-R children (χ^2 for trend = 7.23, df = 1, p = 0.0072; L4/L4 *vs* S4/L4 + S4/S4: χ^2 = 6.49, df = 1, p = 0.0109; L4/L4 *vs* S4/L4: χ^2 = 4.83, df = 1, p = 0.0279), justifying forming two NETpPR genotype groups for an epistasis study: L4/L4 *vs* S4/L4 + S4/S4. NETpPR data for 16 AN-BP trios in the current study (14 trios genotyped for Urwin *et al* (2002) plus two trios recruited and genotyped later) were (transmitted : nontransmitted) alleles L4 (22 : 28), S4 (10 : 4) and genotypes L4/L4 (7 : 12), S4/L4 (8 : 4), S4/S4 (1 : 0) suggesting preferential transmission of S4. Accordingly, AN-R and AN-BP data have been analyzed together and separately in the current report.

Statistical Analysis

The TDT detected preferential transmission of 5-HTTLPR alleles from heterozygous parents and was performed using McNemar's χ^2 test. Hardy–Weinberg equilibrium and epistasis were assessed with χ^2 or Fisher's exact tests. GraphPad InStat V3.05 was used for the unpaired *t*-test. Power calculations were performed using published equations (Risch and Merikangas, 1996), assuming allele frequencies of L = 0.57 and S = 0.43 observed in an Australian population study (Jorm *et al*, 1998). The required significance level was set at 0.05. Two-sided *p*-values were used.

RESULTS

The 5-HTTLPR genotyping data reported here includes those from the same 101 trios involved in Urwin *et al* (2002) in which their NETpPR genotypes were determined. The NETpPR genotype of each AN child in these 101 trios is used in the epistasis analysis below. Additionally, 5-HTTLPR and NETpPR genotyping data from five trios recruited after Urwin *et al* (2002) are also analyzed here (ie a total of 106 trios).

In the 5-HTTLPR association study, there were 106 trios. As the rare 15-repeat allele was detected in only four parents (two East Asian, two Caucasian), it was firstly analyzed as an S allele (data reported here) and then as an L allele (data not shown), with no change in the statistical significance. Genotype frequencies were in Hardy–Weinberg equilibrium (parents: χ^2 = 0.44, df = 1, p = 0.508; AN children: χ^2 = 0.64,

Table 1 Epistatic Interaction Analysis: 5-HTTLPR TDT Data from 106 AN Family Trios Stratified by (Conditioned on) AN Child's NETpPR Genotype

| Child's NETpPR genotype | Number of trios | Allele transmitted from 5–HTTLPR S/L parents | | TDT McNemar's | |
		S	L	χ^2	P
L4/L4	65	26	30	0.29	0.593*
S4/L4+S4/S4	41	24	21	0.20	0.655*
Combined	106	50	51		

*Not significant.

df = 1, p = 0.423). Alleles were (transmitted : nontransmitted) S (102 : 103) and L (110 : 109), and genotypes were (transmitted : nontransmitted) S/S (22 : 26), S/L (57 : 50), and L/L (27 : 30). The TDT ('Combined' data from Table 1) provided no evidence of preferential transmission of either allele as S/L parents transmitted 50 S and 51 L alleles (McNemar's χ^2 = 0.01, df = 1, p = 0.921, odds ratio 1.0, 95% CI 0.7−1.5). This finding was supported by the haplotype-based haplotype relative risk (HHRR) test (Terwilliger and Ott, 1992) as neither allele was significantly more common in patients than in parents (χ^2 = 0.01, df = 1, p = 0.923, odds ratio 1.0, 95% CI 0.7−1.4), and through the haplotype relative risk (HRR) test (Falk and Rubinstein, 1987) as neither the L/L nor S/S + S/L genotype group was significantly more common in patients than in nontransmitted genotypes (χ^2 = 0.22, df = 1, p = 0.642, odds ratio 1.2, 95% CI 0.6−2.1).

Additionally (data not shown), the TDT did not demonstrate preferential transmission of either allele in 101 trios in which the affected child was female (F-AN trios) (McNemar's χ^2 = 0.01, df = 1, p = 0.919, odds ratio 1.0, 95% CI 0.7−1.6), in 90 AN-R trios (McNemar's χ^2 = 0.05, df = 1, p = 0.829, odds ratio 1.0, 95% CI 0.6−1.5), or in 16 AN-BP trios (McNemar's χ^2 = 0.07, df = 1, p = 0.796, odds ratio 1.1, 95% CI 0.4−3.7). The unpaired *t*-test (data not shown) showed no difference between genotype groups (L/L *vs* S/S + S/L) in the 106 trios for age of onset (t = 1.238, df = 104, p = 0.219) or minimum BMI (t = 1.187, df = 104, p = 0.238), in F-AN trios (age of onset: t = 1.177, df = 99, p = 0.242; minimum BMI: t = 0.906, df = 99, p = 0.367), in AN-R trios (age of onset: t = 0.895, df = 88, p = 0.373; minimum BMI: t = 0.846, df = 88, p = 0.400), or in AN-BP trios (age of onset: t = 0.250, df = 14, p = 0.806; minimum BMI: t = 1.027, df = 14, p = 0.322). Allele transmissions from S/L parents in the current and German study (Hinney *et al*, 1997) were homogeneous (χ^2 = 0.08, p = 0.777), justifying combining the results (total = 161 trios) to allow 80 and 99% power to detect a GRR of 1.6 and 2.0, respectively. Of 153 S/L parents in 161 trios, 77 transmitted S and 76 transmitted L (McNemar's χ^2 = 0.01, df = 1, p = 0.936, odds ratio 1.0, 95% CI 0.7−1.4), clearly providing no evidence of association with a 5-HTTLPR allele at these GRRs.

We then investigated possible epistatic interaction between the 5-HTTLPR and the NETpPR polymorphism by stratifying the 5-HTTLPR TDT data based on the AN child's NETpPR genotype (justified in Molecular Genetic

Methods) to form a 2×2 contingency table (Table 1). The top stratum consisted of 5-HTTLPR TDT data from parents of 65 NETpPR-L4/L4 children. The bottom stratum was composed of 5-HTTLPR TDT data from parents of 41 NETpPR-S4/L4 or S4/S4 children. TDT analysis of each stratum provided no evidence of either 5-HTTLPR allele being preferentially transmitted when conditioned on the child's NETpPR genotype. Comparing the strata was not statistically significant ($\chi^2 = 0.48$, df = 1, $p = 0.490$), suggesting no interaction between the 5-HTTLPR and NETpPR polymorphisms in AN. The findings were similar (data not shown) for the F-AN trio data ($\chi^2 = 0.53$, df = 1, $p = 0.465$), AN-R trio data ($\chi^2 = 0.0002$, df = 1, $p = 0.989$) and AN-BP trio data (Fisher's exact test $p = 0.442$).

DISCUSSION

We feel that our results demonstrate that there is no association between 5-HTTLPR and AN in Australians (unless AN is weakly associated with a 5-HTTLPR allele). This finding is similar to results from Germany (Hinney *et al*, 1997), although our study has greater power due to almost double the sample size. If 5-HTTLPR predisposes an individual to developing AN, then its independent effect is extremely small and possibly clinically irrelevant. It must be made clear, however, that negative association of AN with 5-HTTLPR does not necessarily extend to the entire *SERT* gene given the distance from 5-HTTLPR to the intron 2 variable number of tandem repeats (VNTR) polymorphism and 3' UTR. There may be other functional polymorphisms in the *SERT* gene involved in AN that are not in linkage disequilibrium with 5-HTTLPR. A lack of linkage disequilibrium between the ends of a gene is demonstrated in the dopamine transporter gene (Greenwood *et al*, 2002) and dopamine 2 receptor (DRD2) gene (Gelernter *et al*, 1998). Recently, Kim *et al* (2002) also reported that autistic disorder may have stronger association at locations other than at 5-HTTLPR. As intron 2 VNTR is reported to be a functional polymorphism (Lovejoy *et al*, 2003), studies of intron 2 VNTR and haplotypes including 5-HTTLPR and intron 2 VNTR (and other polymorphisms contributing to haplotype blocks in the *SERT* gene) are the minimum required to exclude major effects at this gene in AN (although major effects in complex genetic disorders are unlikely). We also did not observe the increased *S* allele frequency seen in case–control study AN patients possibly due to clinical and/or genetic heterogeneity, variability of linkage disequilibrium between the 5-HTTLPR and another susceptibility allele in different samples, or population stratification affecting the case–control findings. Supporting population stratification, *S* allele frequencies in case–control study controls (Di Bella *et al*, 2000; Sundaramurthy *et al*, 2000; Fumeron *et al*, 2001) and in the nontransmitted alleles reported here range from 0.36 to 0.49 ($\chi^2 = 9.48$, df = 3, $p = 0.024$) and *S* allele frequencies range from 0.11 to 0.70, worldwide (Gelernter *et al*, 1999). We used the TDT to diminish problems due to population stratification. In the TDT, the transmitted (case) and nontransmitted (control) alleles form the parent's genotype and hence are ethnically matched. Interestingly, AN patients have similar *S* allele frequencies across both the current and case–control

studies: 0.48–0.51 ($\chi^2 = 0.60$, df = 3, $p = 0.895$). We also found no association between the *S* allele and AN-BP reported by Di Bella *et al* (2000), although our 16 AN-BP trios provide low power for this observation. Additionally we found no association between 5-HTTLPR and AN females, AN subtype, age at onset, or minimum BMI.

Epistatic interaction between 5-HTTLPR and NETpPR polymorphism was not evident in our AN sample as alleles transmitted from 5-HTTLPR *S/L* parents were not conditional on the NETpPR genotypes of their children. Our results cannot be influenced by linkage disequilibrium as the *SERT* and *NET* genes are on different chromosomes (Brüss *et al*, 1993; Ramamoorthy *et al*, 1993). However, our findings do not necessarily extend to the entire *SERT* and *NET* genes.

In conclusion, although 5-HTTLPR affects reuptake of 5-HT by the 5-HT transporter, we provide no evidence that susceptibility to AN is modified by this mechanism alone, nor in concert with unknown functional mechanisms modified by the NETpPR polymorphism. However, we cannot exclude the possibility of a relation between 5-HTTLPR (and other *SERT* gene variation) and response to SSRIs in AN.

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