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BRIEF COMMUNICATION

Bipolar disorder and the serotonin transporter gene: a family-based association study

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ABSTRACT

Background. The human serotonin transporter gene (5-HTT) is a strong candidate for involvement in the pathogenesis of mood disorders. Two common polymorphisms have been identified in the gene: a VNTR in intron 2 and a functional deletion/insertion in the promoter region. In previous studies we proposed that allele 12 of the VNTR might increase susceptibility for bipolar disorder.

Methods. We have genotyped 122 parent–offspring trios of British Caucasian origin where the proband had DSM-IV Bipolar I disorder (BPI). The results were analysed with the transmission/disequilibrium test (TDT), which examines whether particular alleles are preferentially transmitted from heterozygous parents to affected offspring.

Results. The 12 repeat in the VNTR in intron 2 was transmitted 72 times and not transmitted 56 times ($\chi^2 = 2{\cdot}0$, 1 df, $P = 0{\cdot}16$). If we exclude 24 families in which the proband was a case in our published case–control studies (Collier *et al.* 1996*a*; Rees *et al.* 1997), the excess transmission of allele 12 reaches conventional levels of statistical significance: $\chi^2 = 3{\cdot}85$, 1 df, $P < 0{\cdot}05$. The deletion/insertion polymorphism in the promoter region was not associated with BPI: 66 parents transmitted the inserted (L) allele and 59 parents transmitted the deleted (S) allele ($\chi^2 = 0{\cdot}39$, 1 df, $P = 0{\cdot}53$).

Conclusions. The 12 repeat of the VNTR in intron 2 of the serotonin transporter gene might be a susceptibility factor in bipolar affective disorder. The genetic effect, if true, is likely to be small, and requires confirmation in further studies using parental controls.

INTRODUCTION

Family, twin and adoption studies provide strong evidence that genetic factors are important in the aetiology of bipolar disorder, however, the mode of inheritance is complex and non-Mendelian (Craddock *et al.* 1995). Several lines of pharmacological, neurobehavioural and therapeutic evidence implicate serotonin (5-HT) in the pathogenesis of mood disorders (Meltzer, 1989; Goodwin & Jamison, 1990). The serotonin transporter (5-HTT) reuptakes serotonin into the pre-synaptic neuron (Rudnick & Clark, 1993). Most antidepressants block the action of 5-HTT and can also induce mania in susceptible individuals (Ramamoorthy *et al.* 1993). The gene encoding 5-HTT has been cloned and maps to chromosome 17q11.1-q12 (Ramamoorthy *et al.* 1993; Lesch *et al.* 1994). Two common polymorphisms have been described in the gene: a deletion/insertion of 44 bp in the promoter region approximately 1 kb upstream of the transcription site (5-HTTLPR) (Heils *et al.* 1996) and a Variable-Number-Tandem-Repeat (VNTR) containing 9, 10 or 12 copies of a 16–17 bp repeat element located in

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intron 2 (5-HTT-VNTR) (Lesch *et al*. 1994; Battersby *et al*. 1996).

The promoter polymorphism influences transcriptional activity and 5-HTT function (Collier *et al*. 1996*b*; Heils *et al*. 1996), with the long allele (L) having ~2·5 times higher transcriptional activity than the short one (S). Furthermore, the 5-HTTLPR is unique to humans and primates (Lesch *et al*. 1997), suggesting that it could be important in a higher level of regulation of emotions. This makes 5-HTT a strong candidate for study in affective disorders.

Previous association studies have produced conflicting reports as to whether variation at 5-HTT influences susceptibility to bipolar disorder. Allele 12 of the VNTR was more common in British patients (Collier *et al*. 1996*a*; Rees *et al*. 1997) and in patients from Japan (Kunugi *et al*. 1997) compared with matched controls. A US study observed increased frequency of 12/12 homozygotes in patients compared with matched controls but this was not replicated in a second sample reported in the same paper (Vincent *et al*. 1999). No such differences were observed in five other studies on European populations (Battersby *et al*. 1996; Stöber *et al*. 1996; Bellivier *et al*. 1997; Furlong *et al*. 1998; Hoehe *et al*. 1998) and one on a Chinese population (Collier *et al*. 1996*b*). Allele 9 was more common in one of these studies (Battersby *et al*. 1996). One large collaborative study examining 5-HTTLPR (Collier *et al*. 1996*b*) found an increase of the short allele in patients, however, the odds ratio was low (1·23) and subsequent studies have been negative (Battersby *et al*. 1997; Kunugi *et al*. 1997; Rees *et al*. 1997; Esterling *et al*. 1998; Furlong *et al*. 1998; Hoehe *et al*. 1998; Vincent *et al*. 1999).

A common problem with case–control association studies is the possibility that population stratification might produce false positive results. This problem can be overcome by using the parents of probands and examining whether one allele is preferentially transmitted from heterozygous parents to affected offspring (the transmission/disequilibrium test (TDT), Spielman *et al*. 1993). We have genotyped both polymorphisms at 5-HTT on a new sample of parent–offspring trios in which each offspring has DSM-IV BPI, and analysed the results using the TDT.

METHOD

Sample description

Probands were collected through systematic screening of Lithium Clinics at the Maudsley Hospital, Epsom General Hospital, Royal South Hants Hospital (Southampton), Sheffield General Hospital, and most centres in South and West Wales. Over 1800 individuals were screened with this method. In addition, nine families responded to advertisements in the press. The sample includes 28 families collected by N.C.'s team for an affected sibling-pair linkage study. This team had screened, in addition to South Wales, clinics in the West Midlands and the South of England. The same methods were used by all investigators. Only White Caucasians of European descent were included in the study. All patients gave informed consent for participation in genetic linkage and association studies. Ethics Committee approval was obtained in all local health authorities where patients were recruited.

Consensus best-estimate DSM-IV diagnosis was made on the basis of all available information: personal interview with the SCAN instrument (Wing *et al*. 1990), reports from the parents and hospital records. The OPCRIT checklist was completed for all probands (McGuffin *et al*. 1991; Craddock *et al*. 1996).

The sample included 122 families. There were 55 male and 67 female probands with a mean age of 33·8, S.D. = 8·4 years. Because some probands had been reported in our previous case–control studies (Collier *et al*. 1996*a*, *b*; Rees *et al*. 1997), we also report the genotyping results after excluding the families of these probands from the analysis.

Genotyping

For 5-HTT-VNTR, target DNA was amplified by polymerase chain reaction (PCR) using primers 5′-GTCAGTATCACAGGCTGCGAG-3′ (sense) and 5′-TGTTCCTAGTCTTACGCCAGTG-3′ (antisense), which amplify the region of intron 2 containing the 16–17 bp repetitive element. PCR was performed in a reaction volume of 20 μl which included 80 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH 8·0, 6 pmol of each primer, 200 mMol dNTPs, 1 mM $MgCl_2$ and 1·2 units of Taq DNA polymerase (Amersham). Amplification consisted of 94 °C

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Table 1. *Allele frequency distribution in parents, probands and in the 'control' sample (the non-transmitted parental alleles), and TDT results: the number of times each allele was transmitted (T) and not-transmitted (NT) from heterozygous parents*

Allele	Parents	Probands	'Controls'	T	NT	χ^2 TDT	*P*
5-HTT-VNTR	*N* = 244	*N* = 122	*N* = 122				
9	7 (0·014)	5 (0·02)	2 (0·01)	5	2	—	—
10	185 (0·38)	83 (0·34)	102 (0·42)	53	72	2·89	0·09
12	296 (0·61)	156 (0·64)	140 (0·57)	72	56	2·00	0·16
5-HTTLPR	*N* = 240	*N* = 120	*N* = 120				
L	265 (0·55)	136 (0·57)	129 (0·54)	66	59	0·39	0·53
S	215 (0·45)	104 (0·43)	111 (0·46)	59	66	0·39	0·53
Haplotypes	*N* = 222	*N* = 111	*N* = 111				
9/L	6 (0·01)	5 (0·02)	1 (0·005)	5	1	—	—
9/S	1 (0·002)	0 (0)	1 (0·005)	0	1	—	—
10/L	125 (0·28)	53 (0·24)	72 (0·32)	36	55	3·97	0·05
10/S	34 (0·08)	14 (0·06)	20 (0·09)	12	18	1.2	0.3
12/L	115 (0·26)	66 (0·30)	49 (0·22)	56	39	3·04	0·08
12/S	163 (0·37)	84 (0·38)	79 (0·36)	51	46	0·26	0·6

P values are two-tailed and not corrected for multiple testing. Parental genotypes did not show significant departure from Hardy-Weinberg equilibrium ($P = 0{\cdot}15$ for 5-HTT-VNTR and $P = 0{\cdot}45$ for 5-HTTLPR).

for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s and a final extension step of 10 min at 72 °C. The fragment sizes were: 250 base pairs (9 repeats), 267 base pairs (10 repeats) or 300 base pairs (12 repeats).

For 5-HTTLPR, target DNA was amplified by PCR using the primers 5′-GGCGTTGCCG-CTCTGAATGC-3′ (sense) and 5′-GAGGGA-CTGAGCTGGACAACCAC-3′ (antisense). The promoter region is very GC-rich and good amplification was achieved only when dGTP was replaced by 50% dGTP and 50% 7-deazaguanosine. The 30 μl PCR reaction contained 0·5 units Amplitaq Gold™ (Perkin Elmer), 1% of the commercial buffer for Amplitaq Gold, 1·5 mM $MgCl_2$, 10 pmol of each primer, 200 mM dATP, dCTP and dTTP, 100 mM dGTP, 100 mM deaza-GTP, 5% DMSO and 80 ng genomic DNA Touchdown PCR conditions were adopted using primer annealing temperatures of 63 °C and 62 °C at two cycles each, followed by 35 cycles at 61 °C for 80 s and 95 °C for 30 s and a final extension step at 61 °C for 10 min. The fragments sizes were 528 bp and 484 bp. Both polymorphisms were resolved on 1% agarose/2% Metaphor gels (Flowgen, UK) and visualized by ethidium bromide transillumination.

Statistical analysis

Departure from Hardy-Weinberg equilibrium was examined using a χ^2 test. Preferential transmission of alleles from heterozygous parents to affected offspring was analysed by the TDT (Spielman *et al*. 1993). In order to analyse the 5-HTT-VNTR and the exact 2-locus haplotypes we used the likelihood-based Extended TDT (ETDT), which performs the test for markers with multiple alleles (Sham & Curtis, 1995). The magnitude of linkage disequilibrium between the two polymorphisms was estimated as the proportion of the maximum possible disequilibrium (D*) given the allele frequencies (Cox *et al*. 1998). Power calculations were performed by the method of Camp (1997) using a multiplicative mode of inheritance that most closely corresponds with the odds ratios reported in previous positive studies (Rees *et al*. 1997). Our sample had 75% power to detect the effect size for allele 12 of the VNTR proposed by the combined dataset of these studies (heterozygous odds ratio, OR = 1·57; homozygous OR = 2·56).

RESULTS

The allele frequency distributions in parents, patients and 'controls' and the transmission of individual alleles from heterozygous parents are presented in Table 1.

5-HTT-VNTR

We genotyped 122 families. The 12 repeat in the VNTR in intron 2 was transmitted 72 times and not transmitted 56 times from heterozygous

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parents ($\chi^2 = 2{\cdot}0$, 1 df, $P = 0{\cdot}16$). After exclusion of overlaps, this result reaches conventional level of statistical significance ($\chi^2 = 3{\cdot}85$, 1 df, $P < 0{\cdot}05$).

5-HTTLPR

In 120 families genotyped, 66 heterozygous parents transmitted the long allele and 59 transmitted the short allele ($\chi^2 = 0{\cdot}39$, 1 df, $P = 0{\cdot}53$). After exclusion of overlaps the results on 101 families remained the same ($\chi^2 = 0{\cdot}25$, 1 df, $P = 0{\cdot}62$).

Haplotype analysis

The availability of parents in each case allowed us to construct exact haplotypes for the two polymorphisms in 111 of the 122 families. The frequencies of the six possible haplotypes and their transmission from heterozygous parents are presented in Table 1. Consistent with previous studies (Collier *et al.* 1996*b*; Rees *et al.* 1997) the two polymorphisms showed highly statistically significant evidence of linkage disequilibrium ($\chi^2 = 59{\cdot}3$, 1 df, $P < 0{\cdot}000\,001$) with allele 12 of the VNTR being associated with allele S at 5-HTTLPR. However, the strength of the disequilibrium was modest (disequilibrium coefficient, $D = 0{\cdot}088$, and the proportion of the maximum possible disequilibrium, $D^* = 0{\cdot}53$). Haplotype analysis for disease-haplotype association approaches conventional levels of statistical significance (χ^2 for allele-wise TDT = 10·2, 5 df, $P = 0{\cdot}07$) but, consistent with our previous findings (Collier *et al.* 1996*b*; Rees *et al.* 1997) the 12 allele of the VNTR was preferentially transmitted regardless of the presence of a short or long allele at the 5-HTTLPR.

DISCUSSION

The hypothesis that allele 12 is a susceptibility factor for bipolar affective disorder was proposed initially by Collier *et al.* (1996*a*), replicated by our group in another case–control study (Rees *et al.* 1997) and more recently by Vincent *et al.* (1999) in one of the two samples tested by this group. A study from Japan also found the 12 repeat to be significantly increased in patients, but the frequency of allele 12 in Asian populations is much higher and no allele 9 was observed, so comparisons are difficult. In contrast, seven case–control samples have produced negative results, as summarized in the Introduction. The design of the current study is robust to population stratification and is much less likely to produce a type 1 error than case–control samples. We found a trend that allele 12 of the VNTR is preferentially transmitted from heterozygous patients to affected offspring. This trend becomes significant when overlaps with our previous case–control studies are excluded, thus producing a completely independent replication sample. The odds ratios produced from this new sample (heterozygous OR = 1·22, homozygous OR = 2·71) are compatible with those proposed by the combined dataset in our previous studies (heterozygous odds ratio OR = 1·57; homozygous OR = 2·56, Rees *et al.* 1997).

Only one study so far has implicated the insertion/deletion polymorphism as a susceptibility factor for bipolar disorder (Collier *et al.* 1996*b*). It found an excess of the short (S) variant, however, the effect size was small and the statistical significance was modest considering the large sample size. None of the other studies published (as summarized in the introduction) replicated this finding, which suggests that the original study may have been a false positive.

Our results for haplotype frequencies in patients and controls are similar to those reported previously by Collier *et al.* (1996*b*) and Rees *et al.* (1997), which showed the same trend for allele 12 to be more common in bipolar patients regardless of whether the allele at the 5-HTTLPR is short or long. The observation that, of two polymorphisms that are in linkage disequilibrium with each other, only one shows preferential transmission, is consistent with the modest size of the linkage disequilibrium ($D^* = 0{\cdot}53$, possible maximum = 1).

Functional studies have so far implicated only the 5-HTTLPR as a potential susceptibility factor in behavioural or mood disorders, as the long allele had a higher transcriptional activity than the short one (Heils *et al.* 1996; Collier *et al.* 1996*b*). The VNTR in intron 2 has no known functional significance. This is however possible, as the repeat is followed by an activating protein (AP-1) motif, a putative binding site for a transcription factor comprising the heterodimer c-fos/c-jun and thus may play a role in the regulation of 5-HTT expression (Lesch *et al.*

1994). The length of the VNTR itself may regulate transcription of the gene, in analogy with the VNTR at the insulin gene locus IDDM2 (Catignani Kennedy *et al*. 1995) and the tetranucleotide repeat in the first intron of the tyrosine hydroxylase gene (Meloni *et al*. 1998). A recent hypothesis suggests that micro- and mini-satellite polymorphisms play a role in the expression of many genes and may be especially relevant in complex polygenic disorders and behavioural phenotypes (Comings, 1998). Further work is required to determine whether the 5-HTT-VNTR is of direct functional significance or whether it is closely linked to another functional variant.

In conclusion, although our findings for 5-HTT-VNTR do not reach conventional levels of statistical significance, they are in the same direction as previous positive reports. This provides some support for the hypothesis that allele 12 of the 5-HTT-VNTR is a susceptibility factor for bipolar affective disorder but a number of negative studies suggest that if such an association exists, it is a small effect. Further family-based association studies should confirm or reject this association.

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