Supplement 1

METHODS

5HTTLPR assay

The original assays (1) for the 5HTTLPR used PCR primers in the non-repetitive sequences that flanked the 16 repeat elements, which are each comprised of between 19 and 23 base pairs (bp) (1, 2). The PCR products were 528 bp and 484 bp for the L and S alleles respectively. Heils *et al.* (1) reported that the deletion removed 44 bp that spanned repeats VI-VIII (see Figure 1B). This assay proved less than ideal: the PCR is difficult because of the very high GC content, and the long length of the PCR products. In pilot studies we found that these difficulties cause considerable bias towards S allele identification, since the L allele signal is weak so that heterozygotes are frequently mis-scored as S homozygotes (eg., see Figure 1D of 1). This observation has also been made by others (e.g., 3, 4). Moreover, a reassessment of the polymorphism has been performed by several groups (5-9) and has confirmed our observation that the originally reported 44 bp deletion is in fact a 43 bp deletion.

We improved on the assay by redesigning the PCR primers — our replacement primers were L2 and R2 in Figure 1B; others have used similar modifications (e.g., 10). Although much improved, the assay was not entirely satisfactory so we tried designing primers within the repeats themselves. All these primers worked well under our improved conditions (50% deaza-dG, touchdown PCR) and all combinations of the left (Lx) and right (Rx) primers gave the expected products. We chose to continue work with two combinations, assay L3+R3 and L3+R4 shown in Figure 1B. The sizes for the L and S alleles respectively were 197 and 154 bp for L3+R3, and 126 and 83 bp for L3+R4 (Figure 1C). In a pilot experiment for quality control, one plate of 384 DNA samples was amplified independently with these two assays and the L2+R2 combination (which are outside the repeats, see Figure

1B, and gives 404 bp and 360 bp products for the L and S alleles respectively); our results gave 100% concordance between all three assays.

This choice of primers L3+R3 and L3+R4 provided two PCR products which are semi-independent (L3 is shared) and which could be analysed together on a gel. Moreover, the L3+R4 product did not include any repeats which would have interfered with the primer-extension assay for rs25531. The primer products were pooled (one DNA sample from each primer product) and run on a gel together (twofold multiplexing). Further, fourfold multiplexing was achieved by loading a second pair of samples on to the same gel after the first loading had been electrophoresed for 30 minutes; (Figure 1C). One set of four rows each with 24 lanes in one gel tank, allows 384 different DNAs to be run, loaded and scored in about one hour. In operation, a 384-well plate of DNAs can be amplified for both products and pooled in a way to allow duplicate scoring on two gels. Our pilot experiments showed that this was an efficient and robust system to genotype this difficult polymorphism.

SNP rs25531 assay

The genotyping assay for SNP rs25531 [A/G], which occurs in the long allele of 5HTTLPR, relied on the apparent single-nucleotide difference between repeats VI and VIII: a C-tract that occurs in both repeats after the central TGCA motif (see Figure 1B), has been reported in numerous papers and Genbank to be 6 in repeat VI and 5 in repeat VIII. The deletion of the 43 bp (i.e., as in the S allele) creates a new repeat (consists of the left half of VI and the right half of VIII) in which this C-tract is from repeat VIII. We designed a modified primer-extension reaction (ddATP as the sole dideoxynucleotide) which would readily and clearly distinguish this difference (i.e., 5 versus 6 C's) using the Sequenom MassArray system. [Note, using the PEX1 primer (gcatccccccctgcaccccc[a/g]; see Figure 1B), some of the other repeats could have interfered with this assay, but this is not the case

with the L3+R4 product which spans only repeats V thru IX (Figure 1B)]. Our results, however, indicated that the sequences of the C-tracts of these two repeats had identical 5 Cs. In addition, the rs25531 polymorphism has been assigned to repeat VI (9) as shown in Figure 1B.

A re-analysis of our extension products with the PEX1 primer revealed that our assay does in fact detect the G allele of SNP rs25531. However, due to the 100% identity in repeats VI and VIII (and therefore also in the VI-VIII hybrid repeat in the S allele) of the 14 nucleotides at the 3' end of the PEX1 primer (seen underlined in the PEX1 primer; gcatccccccctgcaccccc[a]), there will always be some 'A' allele extension product. That is, L_GL_G individuals will still be genotyped as L_GL_A due to the 'A' in repeat VIII and hence, it is not possible to differentiate between L_GL_G and L_AL_G genotypes. We also note that there is an A/G variant at the equivalent position in the VIII repeat which would also be scored by our rs25531 assay. The variant S_AS_G is, however, extremely rare (0.1%) and has only been found in Asian samples (9). Out of a total of 6258 individuals genotyped for both 5HTTLPR and rs25531, only 14 were of the unexpected genotype S_AS_G (0.2%). None of these reported having non-European grandparents and so the 5HTTLPR and rs25531 genotypes were set to missing for these individuals. Five two-locus 5HTTLPR-rs25531 genotype classes: SS, SL_G, SL_A, L_GL_A and L_AL_A can be made, after removal of S_G genotypes. Based on the relative frequencies of SL_G and SL_A we estimate that only 0.9% of the total sample is expected to have genotype L_GL_G, or 12% of those genotyped as L_GL_A.

Quality control checks on genotype data

Our study design allowed us to undertake quality control checks often not possible in association studies. In total, both twins of 857 MZ pairs were genotyped. MZ pairs were identified, on the whole, by self-report, but previous genotyping has refined the zygosity

diagnosis. The overall rate of genotype discordance was 0.16%. Duplicate samples were genotyped for 764 individuals with an overall rate of genotyped discordance of 0.45%. For the CIDI sample, 29% of participants had both parents genotyped, and 50% had one parent genotyped. Genotyping from other studies (11, 12) had confirmed the pedigree relationship between samples. 32 Mendelian errors were detected, mostly from 5HTTLPR and rs25531.

Selection of unrelated individuals

Ascertainment into the CIDI sample was based on concordance or discordance of neuroticism scores, thus distorting the within family variance for this trait and anything correlated to it. Therefore, association analysis for quantitative traits used one individual per family selecting the individual that deviated most from the population mean for the trait under analysis. In this way, the most informative individual per family is selected. Empirical p-values for association ensured against a possible distortion of the distribution of the test statistic.

Where more than one sibling from a family could qualify for selection as a case, prioritization was given to individuals with a co-morbid diagnosis of anxiety and depression, recurrent major depression, early onset depression, co-morbid diagnosis of different anxiety disorders and highest neuroticism score (listed in order of priority). Both SSAGA and CIDI interviews allowed allocation of the anxiety disorders, social phobia, panic disorder and agoraphobia and the CIDI interview also allowed allocation of generalized anxiety disorder (GAD) and obsessive compulsive disorder (OCD) diagnoses. Controls were selected as unrelated individuals, from families in which no siblings who completed the questionnaires (including those not supplying a DNA sample) received a diagnosis of either major depression or of an anxiety disorder. Within a family, the sibling with the lowest neuroticism

score was selected and SSAGA controls were only selected if their neuroticism scores were less than the population average.

Power and significance

Our combined study sample has over 1000 cases and 1000 controls and more than 80% power to detect causal variants with genotype relative risk of the heterozygotes of 1.4 under a multiplicative model on the risk scale (additive on the log(risk) scale) for genotyped markers of frequency between 0.1-0.9 that are in perfect LD with the causal variant at a significance threshold of 0.05 (13). We use the 5% significance threshold, despite multiple testing, for discussion of results. However, we place more emphasis on SNPs that show evidence of association in the independent SSAGA and CIDI subsets and that show more significant association in the two case subsets, early onset recurrent major depression (212 cases) and depression co-morbid with anxiety (265 cases), that are thought to be genetically more homogenous groups (14, 15) . We used a permutation procedure to test this formally: the sample identities, together with their multiple case-control status allocations were randomly permuted with the genotype sets (so that the linkage disequilibrium between markers is maintained) of all cases and controls. The permuted data sets were analysed in the same way as the real data and the empirical significance level calculated as the frequency of the observed, or more significant, pattern of significance occurring for any of the markers across case-control data sets out of 10,000 permutation replicates, with the real results counting as one replicate.

RESULTS

Association analysis with 5HTTLPR

We observed association ($p < 0.05$) for the 5HTTLPR (L allele) for the CIDI sample, the strongest association being for the Depression cases ($p = 0.008$). The association with rs2020934 (which has $r^2 = 0.49$ with 5HTTLPR) was $p = 0.048$; but we note that in this example 30 cases and 16 controls had missing genotypes for 5HTTLPR. When the same individuals are used, the association with rs2020934 was $p = 0.021$ and the association with the two SNP proxy was $p = 0.028$ for the CA haplotype which tags the S allele and $p = 0.025$ for the CG haplotype which tags the L allele. The association of 5HTTLPR-rs25531 with Depression in the CIDI group was less significant ($p = 0.034$) than for 5HTTLPR alone. If we assume that the 0.9% misclassification of L_GL_G and L_GL_A affects cases and controls equally then the association accounting for the misclassification becomes $p = 0.036$. In contrast, there was no evidence for association with 5HTTLPR or 5HTTLPR-rs25531 in the SSAGA sample, for which any trend in association is with the S allele. The difference between the data sets reflects a significant difference in allele frequency between the SSAGA and CIDI controls samples ($p = 0.007$). Some evidence for association is seen in the CIDI sample for markers 10, 12, 14 and 15 that are in the same LD block as the 5HTTLPR (marker 11), implying that the detected association does not reflect genotyping artefacts and significant ($p < 0.05$) differences in allele frequencies between control samples are also observed for these markers. To determine whether the association in the CIDI sample reflected that it was ascertained to represent more extreme cases than the SSAGA sample (Table 3), we selected an "extreme" SSAGA set of 371 cases with standardised neuroticism score > 0 and 260 controls with standardised neuroticism score < -0.9, but this sample still showed no support for the association, or trend in association, observed in the CIDI sample. We interpret the

association between markers 10-15 (Figure 2) and Depression in the CIDI sample as being a chance result because the association is not replicated in the SSAGA sample.

DISCUSSION

Trying to deduce the LD between rs6354 and rs2020936 and STin2

Zaboli *et al.* (16) genotyped and reported the LD between 5HTTLPR, STin2 and two nearby SNPs (rs140701 and rs1042173) and found that STin2 and the two SNPs were in a different haplotype block to 5HTTLPR. Using CEU genotypes downloaded from the HapMap (17) website, we find that SNPs rs6354 and rs2020936 group in the same |D'| haplotype block as rs140701 and rs1042173 (|*D'*|=1 but $r^2 \approx 0.2$, reflecting the coupling, but different frequencies, of their minor alleles), and the C-C haplotype of rs6354 and rs2020936 is present exclusively on the G-T haplotype of rs140701 and rs1042173. Although Zaboli *et al.* (16) report in their Table IV 5HTTLPR-STin2- rs140701-rs1042173 haplotypes we were not able to deduce the likely coupling of alleles of rs6354 and rs2020936 with STin2.

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