Hardy–Weinberg disequilibrium identified genotyping error of the serotonin transporter (SLC6A4) promoter polymorphism

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We analyzed the putative functional promoter polymorphism of the serotonin transporter (5-HTTLPR) in two large autism spectrum disorder samples and a control sample. A Hardy–Weinberg disequilibrium was detected for 5-HTTLPR in the unaffected founders of both autism spectrum disorder samples and control samples. When we lowered the total magnesium concentration in the polymerase chain reaction below levels reported in previously published studies, we observed a shift in relative allele frequencies and restoration of the Hardy–Weinberg equilibrium. Our data suggest that higher magnesium concentrations caused allele-dependent, non-random genotyping errors. Genotyping data obtained from the 2 mM magnesium protocol increased the significance of linkage and gave suggestive ($P=0.06$) association with autism spectrum disorder, whereas the corrected genotypes of 5-HTTLPR provide no linkage information beyond the results we have previously published and no evidence of association with autism spectrum disorder. We present details regarding appropriate polymerase chain reaction conditions for the accurate genotyping of this polymorphism. *Psychiatr Genet* 16:31–34
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The serotonin transporter (5-HTT) has been suggested as a candidate gene for autism spectrum disorder (ASD) since the early 1960s (Cook and Leventhal, 1996). An insertion/deletion polymorphism that lies within the putative promoter region of the gene for the serotonin transporter length polymorphism repeat (5-HTTLPR) has been intensively studied with regard to its relationship with ASD and many other psychiatric disorders (Glatt and Freimer, 2002). 5-HTTLPR is reported to have an allele-dependent affect on the expression of 5-HTT (Heils *et al.*, 1996). We tested the hypothesis that 5-HTTLPR was associated with ASD in two of the largest populations of ASD families yet to be assembled (Risch *et al.*, 1999; Geschwind *et al.*, 2001). [As previously described (Risch *et al.*, 1999; Geschwind *et al.*, 2001), all human study participants' protection and oversight was provided by the appropriate IRBs.]

Significant linkage was found from the analysis of the original genotype data of 5-HTTLPR (Fig. 1a). The founders of both ASD samples were found to be in Hardy–Weinberg disequilibrium (HWD) (Table 1). A control population was also found to be in HWD for 5-HTTLPR (Table 1). As the control population was not affected with ASD, or any other major psychiatric disorders, the finding of HWD cannot be explained by relationship to phenotype. Although other explanations could not be ruled out, this finding suggested the possibility of genotyping error. Summed over the entire sample (founders and controls combined), we detected 5-HTTLPR frequencies for the short and long alleles of 66 and 34%, respectively.

5-HTTLPR is known to be extremely difficult to genotype (Kaiser *et al.*, 2002 and personal communications; L.A. Weiss, J.S. Sutcliffe, E.H. Cook). We were unable to successfully generate a polymerase chain reaction (PCR) product using the published protocols and, instead, adapted these protocols until we obtained a reliable PCR product. The results of this modified protocol were interpreted consistently by three independent researchers (error rate $<0.1\%$ for 1248 genotypes). HWD of the genotypes, especially in the control sample, led us to suspect genotyping error. We discovered that when the total magnesium (Mg) concentration was lowered from 2.0 to 1.0 mM, many genotypes changed and the Hardy–Weinberg equilibrium (HWE) was restored in all three samples (Table 1). The 5-HTTLPR short allele frequency shifted from 66 to 46% when using the lower Mg protocol, which is consistent with published studies that range from 36 to 59% (Cook *et al.*, 1997; Klauck *et al.*, 1997; Yirmiya *et al.*, 2001; Kim *et al.*, 2002; McCauley *et al.*, 2004).

For all PCR assays, we used identical primers {HTTAL; 5′-Fam-CGC TCT GAA TGC CAG CAC CTA ACC-3′

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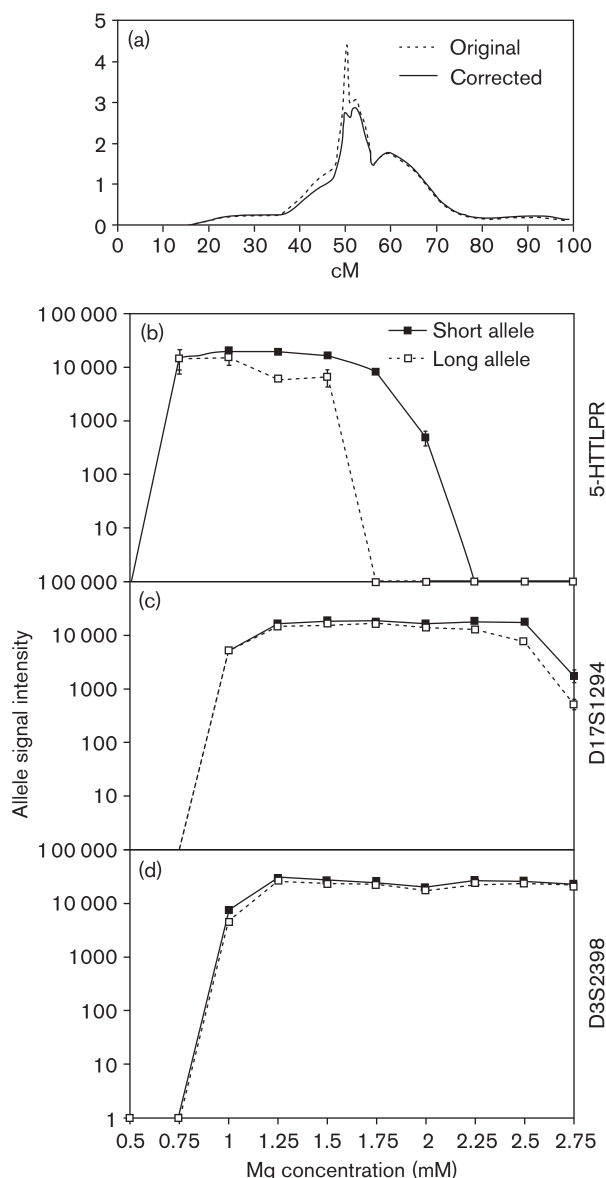
Fig. 1



(a) Multipoint linkage analysis results on chromosome 17 [maximum logarithm of odds (LOD) score (MLS) calculated using the Mapmaker/Sibs program (v 2.1) that is part of the GENEHUNTER 2.1 software package]. The dashed line shows the MLS when calculated using the original erroneous genotypes of serotonin transporter (5-HTTLPR). The solid line shows the MLS when using the corrected genotypes of 5-HTTLPR. The solid line is virtually identical to the linkage results calculated without adding in the 5-HTTLPR (Yonan *et al.*, 2003), demonstrating that this polymorphism provides no additional linkage information. (b) The effect of titrating Mg in the polymerase chain reaction (PCR) protocol on 10 heterozygous individuals for 5-HTTLPR, and two microsatellites, (c) D17S1294 and (d) D3S2398. 5-HTTLPR shows a clear bias towards amplifying the short allele over the long allele, leading to the possible miscalling of these heterozygous individuals as homozygous short. The *X*-axis shows the final concentration of Mg used in the PCR protocol (the touchdown amplification protocol was used for Mg concentrations 1.5–3.0 mM; the amplification protocol without the touchdown was used for Mg concentrations 0.5–1.5 mM). The *Y*-axis shows the intensity of each allele's peak on a log scale read from an ABI 3730XL sequencer. The dashed line with open boxes represents the long allele of each polymorphism. The solid line with solid boxes represents the short allele.

and HTTBL; 5′-GGG ATT CTG GTG CCA CCT AGA CGC-3′ that amplify a 415(short or S allele)/459(long or L allele) base pair product. The original PCR protocol contained 50 ng of DNA [all DNA samples were diluted in 1 × Tris-HCL/ethylenediaminetetraacetic acid (EDTA) (TE) buffer for storage, which contains 1.0 mM EDTA], 1 × Roche (Roche Applied Science, Indianapolis, Indiana, USA) FastStart PCR buffer, 0.2 mM deoxyribulase nucleotide (dNTP) mix, 2.0 mM $MgCl_2$, 1 × GC-RICH (a region of DNA sequence that is rich in G and C nucleotides) solution, approximately 0.4 units of Roche FastStart Taq polymerase and 0.25 μM concentration of each primer in 10 μl total volume. Samples were amplified using a 'touchdown' protocol; 95° for 5 min followed by a 13-cycle two-step touchdown with a 15-s 94° denaturing step and a 1-min annealing step that begins at 72°, and decreases 0.5° per cycle; followed by 35 cycles consisting of 15 s at 94°, 15 s at 66° and 45 s at 72°, and a final extension step at 72° for 2 min.

The lower Mg protocol is identical, except that the final concentration of $MgCl_2$ is 1.0 mM rather than 2.0 mM and a different amplification procedure (without the touchdown) was used: 95° for 5 min, followed by 35 cycles consisting of 30 s at 95°, 30 s at 60° and 1 min at 72°, with a final extension step at 72° for 7 min. We note that our experiments control for 'total' (i.e. 'free' plus 'bound') magnesium concentration and that 'free' magnesium concentration will reflect a more complicated set of interactions with the chelating agent EDTA and with levels of dNTP (provided as a salt compound that will bind with free magnesium) that will change throughout the PCR amplification assay. Nonetheless, by holding all assay variables constant and varying only total magnesium concentrations, our data show that magnesium concentration affects PCR amplification in a primer-dependent manner. A relatively extensive literature describing the effect of magnesium concentration on PCR amplification reactions exists (see Fugger *et al.*, 1990).

The Mg concentration was first titrated without changing the amplification protocol. At Mg concentrations of 1.75 mM and above, however, products can only be produced using the touchdown amplification protocol. At Mg concentrations of 1.25 mM and below, PCR products are only produced using the modified amplification protocol shown above, with no touchdown. At the Mg concentration of 1.5 mM, both amplification protocols worked and were 100% consistent in the genotypes produced. It is interesting to note that one amplification protocol (without touchdown) worked consistently at any Mg concentration tested for the two independent microsatellites that were also genotyped in this study (Fig. 1c and d).

We examined the genotypes that changed when the Mg concentration was decreased. The single largest change was the conversion of homozygous short (SS) genotypes

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Table 1 Genotype frequencies by protocol

	Original protocol			Low Mg^{2+} protocol		
	AGRE	Stanford	Controls	AGRE	Stanford	Controls
Genotype frequencies						
SS	241	101	134	86	51	51
SL	204	71	68	293	100	115
LL	86	33	20	146	51	54
Significance of HWD	0.00004	0.001	0.003	NS	NS	NS

Comparison of the genotypes, divided by population, depending on which polymerase chain reaction protocol was used. The *P*-values of the Hardy–Weinberg calculations are shown below the genotype frequencies for each population, a significant *P*-value is evidence that those genotypes are in Hardy–Weinberg disequilibrium (HWD). All NS *P*-values are equal to or greater than 0.5. AGRE, Autism Genetic Resource Exchange.

to heterozygous (SL) (68% of genotypes that changed), revealing a bias towards amplifying the short allele over the long allele when using the higher Mg protocol. This change in genotype was further examined by a titration of the Mg concentration on 10 individuals heterozygous for 5-HTTLPR, shown in Fig. 1b. At low Mg concentrations, the SL genotype is accurately reflected in signal intensity; however, as the Mg concentration increases, the short allele is preferentially amplified. At high Mg concentrations (1.75 or 2.0 mM), no long allele product was detected. These heterozygous individuals would have been erroneously genotyped as SS at these Mg concentrations. Although published genotyping protocols vary for Mg concentrations, most use Mg concentrations from 1.5 to 2.5 mM (Cook *et al.*, 1997; Kim *et al.*, 2002; McCauley *et al.*, 2004).

In order to test whether the allele-specific sensitivity to Mg was particular to 5-HTTLPR or was instead found whenever genomic DNA heterozygous for any length polymorphism was amplified, the titration of Mg concentration was repeated with two microsatellite markers on 10 individuals heterozygous for these markers. Figure 1c and d shows that both these microsatellites are much more robust to the changes in Mg concentration than 5-HTTLPR.

The linkage and association analyses were repeated using the new genotypes obtained from the low Mg protocol. While the biased 5-HTTLPR genotypes increased the linkage score dramatically, the corrected genotypes of 5-HTTLPR provided no additional indication of linkage in this region compared with the surrounding microsatellites alone (Yonan *et al.*, 2003; Fig. 1a). Evidence of association between 5-HTTLPR and ASD also vanished with the modified protocol (data not shown). These findings are curious, given the evidence that both cases and controls are affected by this systematic genotyping error; however, it has been previously reported that removing studies (in a meta-analysis) where the genotypes of 5-HTTLPR were not in HWE decreased the evidence for association between 5-HTTLPR and anxiety-related personality traits (Munafo *et al.*, 2004). These results are especially intriguing when noting that the reports for association between autism and 5-HTTLPR have been contradictory, with some studies reporting preferential transmission of the short allele (Cook *et al.*, 1997; McCauley *et al.*, 2003; Conroy *et al.*, 2004) and others reporting preferential transmission of the long allele (Klauck *et al.*, 1997; Yirmiya *et al.*, 2001).

These results emphasize the importance of determining HWE. Literature reporting an allelic association between 5-HTTLPR and autism clearly shows evidence of HWE (Cook *et al.*, 1997; Kim *et al.*, 2002; McCauley *et al.*, 2003), which suggests that their genotypes were accurate without lowering the Mg concentration. Our results, however, point out the particular sensitivity of 5-HTTLPR to Mg concentration. We have found evidence that the long allele of 5-HTTLPR amplifies poorly at higher Mg concentrations, leading to non-random genotyping errors that can appear to show significant linkage or association between genotype and disease. As previously reported, future studies should carefully examine the PCR conditions used when genotyping this polymorphism (Kaiser *et al.*, 2002) and, as with any bi-allelic polymorphism, HWE should be evaluated in both case and control samples. Finally, we note that validity of the HWE test presumes that the sample is adequately powered to detect deviations among the allelic variants; thus, while deviation from HWE will warn investigators to check for assay bias, acceptance of the null hypothesis may not mean that allele dropout has not occurred.

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