

ORIGINAL ARTICLE

The imprinted gene *LRRTM1* mediates schizotypy and handedness in a nonclinical population

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Imprinted genes have been posited to have important roles in human brain development and cognition, but their effects in nonclinical populations have yet to be investigated. Single-nucleotide polymorphisms (SNPs) of the imprinted gene *LRRTM1* have previously been associated with schizophrenia risk and with handedness in individuals with dyslexia. We tested the hypothesis that genetic variation (SNPs) and epigenetic variation (methylation) in this gene are associated with schizotypy and handedness in a nonclinical population. Risk alleles of the three schizophrenia-linked SNPs were associated with significantly and substantially higher levels of total schizotypy. Variation in SNP genotypes was not associated with handedness, but levels of methylation in a block of CpG sites in the putative *LRRTM1* promoter region were associated with more-mixed handedness. These findings provide evidence of continuity between schizophrenia and schizotypy with regard to the psychological effects of allelic variation in this imprinted gene, and show that epigenetic variation in an imprinted gene mediates the development and expression of human handedness.

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INTRODUCTION

Imprinted genes are expressed according to their parent of origin, the mother or father.¹ Evidence from mouse knockouts, and human disorders such as Angelman and Prader–Willi syndromes, indicate that such genes influence aspects of brain development, cognition, and behavior.^{2,3} However, the effects of genetic and epigenetic variation in imprinted genes with predominant effects in the brain have yet to be investigated among healthy human populations, aside from one study showing that schizophrenia-associated single-nucleotide polymorphisms (SNPs) in the imprinted gene *GABRB2* mediate levels of self-reported altruism.⁴

The brain-expressed imprinted gene *LRRTM1* encodes a leucine-rich repeat transmembrane protein that interacts with neuroligins and neuroligins to modulate synaptic cell adhesion in glutamatergic neurons.^{5–7} Francks *et al.*⁷ and Ludwig *et al.*⁸ have reported associations of SNPs and haplotypes in *LRRTM1* with two strongly associated phenotypes, schizophrenia and handedness,^{9–16} and two studies of mouse knockouts for *LRRTM1* have identified effects on social behavior.^{17,18} Associations of specific genes and SNPs with both schizophrenia and handedness are important because schizophrenia (as well as schizotypy) have been widely linked with reduced cerebral lateralization (meta-analysis by Somers *et al.*¹³) and its strong proxy of mixed or left handedness.^{9–15,19,20} Indeed, Berlim *et al.*²¹ hypothesized that handedness, brain asymmetry, and schizophrenia share an underlying genetic basis. Although epigenetic

variation, such as CpG methylation that modulates gene expression, has been postulated as an important causal factor in cerebral lateralization and schizophrenia risk,²² it has yet to be investigated in this context.

In this study, we genotyped the three *LRRTM1* SNPs that have been previously linked with schizophrenia and handedness, and analyzed methylation at 19 CpG sites in the putative *LRRTM1* promoter region using pyrosequencing. Based on the findings of Francks *et al.*⁷ and Ludwig *et al.*,⁸ it was predicted that (1) risk alleles from the schizophrenia-risk haplotype will be associated with higher total schizotypy score; (2) individuals with these risk alleles will show a higher incidence of left or mixed handedness; and (3) methylation levels in the CpG island will influence total schizotypy and handedness.

MATERIALS AND METHODS

Psychometric measures

Schizotypy was measured using the Schizotypal Personality Questionnaire-Brief Revised (SPQ-BR²³). Data are also reported from the three subscales of the SPQ-BR, 'Interpersonal', 'Cognitive Perceptual', and 'Disorganized', in order to localize cognitive effects of genetic and epigenetic variation. As there was no *a priori* expectation for associations with the SPQ-BR subscales, Benjamini and Hochberg's²⁴ false discovery rate adjustments were applied to reduce the risk of type I error for these tests. Handedness was measured with the 32-item Waterloo Handedness Questionnaire (WHQ²⁵). The score for each WHQ item

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ranges from +2 for strong right, +1 for weak right, 0 for ambidextrous, or -1 for weak left and -2 for strong left; as such the total scale ranges from +64 to -64. To be consistent with Francks *et al.*,⁷ strength of handedness was used by taking the absolute value of the handedness score such that values near 0 represent mixed handedness and values near 64 represent strong handedness (right or left). Owing to the skewed distribution of absolute handedness, with the majority of participants being strongly right handed, Spearman correlation tests were used where applicable (rather than Pearson's correlation). Handedness was also assigned as self-reported right, left, or ambidextrous for some tests.

Samples

The psychometric measures were tested in questionnaire form for a large sample of Caucasian undergraduate students at both the University of Alberta (Edmonton, Canada) and Simon Fraser University (Burnaby, Canada). DNA was collected, extracted, and genotyped from 554 individuals (357 females and 197 males) as previously described,²⁶ with buccal samples as the source of all DNA. Although methylation levels of some genes can be more or less tissue specific, previous psychiatric-genetic studies have successfully used saliva or peripheral blood cell samples to quantify methylation of brain-expressed genes (for example, study by Nohesara *et al.*²⁷). A subset of individuals (29 females and 16 males) was selected for epigenetic data collection, so as to include as many left-handed and mixed-handed individuals as possible. All protocols were carried out according to guidelines established by the ethics boards of both universities.

Genetic data

The three SNPs (rs1007371, rs1446109, and rs723524) from the haplotype previously shown to be associated with handedness and schizophrenia were genotyped.⁷ These SNPs occur on 2p12 in a region of strong linkage disequilibrium (LD), 137 kb upstream of *LRRTM1* and including the predicted promoter.⁷ Genotyping was performed by Genome Québec (Montréal, Canada). Genotypes were scored using a recessive inheritance model, based on the findings of Francks *et al.*⁷ in which the risk genotype comprises two copies of the minor allele. Unlike Francks *et al.*⁷ and Ludwig *et al.*,⁸ parental genotype data were not available, so parent-of-origin effects could not be analyzed. This absence of parental data renders our analyses relatively conservative with regard to tests for association of genotypes with schizotypy and handedness.

Epigenetic data

The larger (1169 bp) *LRRTM1* CpG island tested by Francks *et al.*⁷ (hg19 assembly, chr2:80,529,678-80,530,846) was selected to quantify variation in levels of methylation. As CpG islands appear to be relatively uniformly methylated or unmethylated, a 300-bp region containing 19 CpG sites was chosen for detailed analysis as representative of the larger region.

Bisulfite conversion of ~30 ng DNA was performed using the Sigma-Aldrich Imprint DNA Modification Kit (Sigma-Aldrich Co., St Louis, MO, USA), following the manufacturer's standard protocol. Primers were designed using PyroMark Assay Design software 2.0 (Qiagen, West Sussex, UK) or manually where CpG density resulted in software error. The PCRs were designed to amplify a 1.5-kb region of the CpG island using primers 1-fwd and 3-rev, followed by a nested PCR using 3-fwd and biotinylated 3-rev to amplify a smaller region (Supplementary Table S1). PCR protocols were carried out following the methods of Tost and Gut.²⁸

DNA methylation was quantified at each CpG site by pyrosequencing using the Pyromark Q24 system (Qiagen, UK) following the manufacturer's standard instructions and the Pyro Q24 CpG 2.0.6 software. The software quantifies the C (methylated) to T (unmethylated) ratio at each CpG site as a percentage and assigns a quality score of blue ('pass'), yellow ('check'), or red ('fail'). CpG sites were labeled according to the order in which they occur along the island. Analyses were restricted to samples that achieved three replicates of either yellow or blue quality, where at least one replicate showed blue quality, and methylation levels used in the analyses were derived by averaging across replicates.

SNP analyses

Associations between the above measures and risk genotypes for each SNP were analyzed using *t*-tests. For SNPs with fewer than 10 individuals belonging to one genotype group, nonparametric Mann-Whitney *U*-tests (MWU) were applied in place of *t*-tests. Similar to Francks *et al.*,⁷ associations were tested in males and females pooled. However, given evidence of sex-dependent imprinting effects at autosomal loci,²⁹ males and females were also analyzed separately.

CpG island analyses

As CpG islands are relatively uniformly methylated or unmethylated,^{30,31} percent methylation was averaged across CpG sites as a proxy for overall methylation level of the island. Pearson's correlation was used to compare schizotypy scores to differences in average methylation. Individuals were also ranked by schizotypy score and split into two groups of high versus low schizotypy in order to perform *t*-tests with average methylation. To test for associations between methylation levels and handedness, Spearman correlations were performed using strength of absolute handedness, as well as analysis of variance using categorical handedness (that is, right versus left versus mixed). The same tests were applied to compare schizotypy score with both measures of handedness.

CpG islands tend to be near transcription start sites and are generally unmethylated; high levels of methylation of CpG sites are associated with transcriptional inactivation.^{32,33} It was therefore assumed that relatively high methylation would indicate reduced expression, which could be related to psychological phenotypes. Differential methylation by parent of origin, in the form of an imprinting control region, has not been identified for *LRRTM1*.

Given evidence showing that methylation at sets of CpG sites may be associated with human phenotypes (for example, study by Wehkalampi *et al.*³⁴), it was predicted that different regions of CpG sites in *LRRTM1* may influence the phenotypic measures analyzed here. Principal component analysis was also performed to reduce the dimensionality of the methylation data and the number of tests, for males and females separately, and pooled. The first two factors (PC1 and PC2) were tested for association with schizotypy and handedness using Pearson's correlations. All statistical analyses were performed using R, version 2.15.1.³⁵

RESULTS

SNP variation in relation to schizotypy

All three SNPs were in Hardy-Weinberg equilibrium ($P > 0.10$). Nine (1.6%) of 554 total individuals were homozygous for the risk haplotype identified by Francks *et al.*⁷ The frequencies found in our sample for the three SNPs analyzed (rs1007371, rs1446109, and rs723524) appear to be representative of the recessive genotype frequencies in European populations (1%, 1.6%, and 4.3%, respectively; dbSNP). Francks *et al.*⁷ reported haplotype frequencies ranging between 7.6 and 12.1%, presumably because the samples included in their study were enriched for cases of schizophrenia. Two of the three SNPs (rs1007371 and rs1446109) were in complete linkage disequilibrium, and were thus analyzed together (as a single marker).

For both sexes pooled, individuals carrying the risk genotypes for the linked SNPs, rs1007371 and rs1446109, scored significantly higher for total schizotypy than did individuals with the non-risk genotypes (risk genotype mean = 92.75, s.d. = 16.4; non-risk genotype mean = 80.82, s.d. = 15.6; MWU test = 1203, $P = 0.033$; Supplementary Table S2). In females only, all three SNPs were associated with significantly higher scores for both total schizotypy and the interpersonal subscale in risk-allele carriers (MWU test, $P < 0.05$), and the two linked SNPs, rs1007371 and rs1446109, were associated with significantly higher scores on the disorganized subscale (MWU test, $P = 0.016$) (Supplementary Table S3). The associations in females alone, however, did not survive correction

for multiple testing by false discovery rate (adjusted P -values > 0.1). No significant association was seen in males, of any of SNPs or the risk haplotype, with measures of schizotypy (Supplementary Table S4). There were no effects of any of the SNPs on absolute handedness in males and females, pooled or separately (Supplementary Tables S2–S4). The statistical significance of some effects in females and both sexes pooled, but not in males, may be due in part to smaller sample sizes for males ($N=197$) than females ($N=357$).

Methylation in relation to schizotypy

Methylation data were collected for 45 individuals (29 females and 16 males) at 19 CpG sites in the CpG island. Average methylation for all samples, across sites, was 4.3% (s.d. = 1.96%). As methylation data for each CpG site were averaged for three pyrosequencing runs, repeatability was calculated for a set of eight of the CpG sites (CpG78–85). Repeatability for each of these CpG sites was high, ranging from 62 to 95%, which indicates that between-individual variation was much greater than within-individual variation.

Average methylation and total schizotypy showed no associations by Pearson's correlation for any of the groups ($P>0.5$). In addition, there was no difference in average percent methylation between individuals scoring high versus low on the SPQ (split in half) by Kruskal–Wallis test for males ($P>0.40$) and females ($P>0.50$) separately or pooled ($P>0.50$).

Methylation in relation to handedness

Average methylation and absolute handedness showed no associations by Spearman correlations for both sexes pooled, for males or for females (all $P>0.10$). The individuals with methylation data were grouped by handedness according to self-report on the questionnaire (Supplementary Table S5) or calculated from the WHQ, where individuals who scored between -29 and $+29$ were categorized as ambidextrous, and those who scored above or below were categorized

as right- or left handed, respectively (Supplementary Table S6). Handedness was also grouped into 'weak', with an absolute handedness score ranging 0–29, versus 'strong' (> 30). For males and females pooled, no association was found between average methylation and categorical handedness by applying a Kruskal–Wallis test ($P>0.40$). Moreover, no significant associations by Kruskal–Wallis test were found in males and females pooled for handedness categorized by WHQ scores ($P>0.50$) or strength of handedness ($P>0.50$). Owing to small sample sizes for some handedness by sex combinations, this test was not performed for males and females separately.

Handedness in relation to schizotypy

For both sexes pooled, schizotypy was not associated with self-reported handedness (Kruskal–Wallis test, $P>0.50$), WHQ-categorized handedness ($P>0.50$), or strength of handedness ($P>0.50$). Similarly, schizotypy was not correlated with absolute handedness (Spearman correlations) for either the pooled sample ($P=0.346$) or females separately ($P>0.50$), but males showed a trend-level negative correlation between handedness and schizotypy (Spearman's correlation, $\rho = -0.432$, $P=0.095$). This negative correlation would be interpretable as more-mixed handed males showing relatively high schizotypy score.

Analyses of CpG sites

For 19 CpG sites within the pyrosequenced region, sites closer together showed more strongly correlated methylation levels than sites farther apart (Mantel test, $r=0.277$, $P=0.01$). Adjacent CpG sites were also more strongly correlated than nonadjacent sites (t -test, $t=4.049$, $P=0.0001$). CpG sites are thus not necessarily independent in their effects because of high intercorrelation.

To reduce the dimensionality of the data set, given the intercorrelation in methylation between CpG sites, principal components analysis was applied to the CpG methylation data. Together, PC1 and PC2 explained 50% of the variance. PC1 showed strong positive loadings, representing strong intercorrelation between sites, especially for the block of CpG sites between 78 and 85 (Table 1). A t -test confirmed that inter-site levels of methylation in the CpG78–85 block were significantly more strongly correlated (mean $r=0.49$) than between sites outside the block (mean $r=0.29$; $t=6.24$, $P=0.0001$). These results indicate that PC1 can be interpreted biologically as

Table 1 PCA loadings for methylation at CpG sites for males and females

CpG site	PC1 loading	PC2 loading
67	+0.1551	+0.0842
68	+0.1635	-0.2456
69	+0.0321	-0.4056
70	+0.1536	-0.1069
71	+0.3193	-0.0354
72	+0.1005	-0.3509
73	+0.1196	-0.4830
74	+0.1594	-0.1631
75	+0.2639	+0.0969
76	+0.2030	-0.3910
77	+0.0651	+0.2218
78	+0.2237	-0.0289
79	+0.1425	+0.1948
80	+0.3092	+0.1821
81	+0.2909	+0.1395
82	+0.3362	+0.1030
83	+0.3324	+0.1115
84	+0.2731	-0.0983
85	+0.3301	+0.1917
Proportion of variance	+0.37	+0.13

Abbreviation: PCA, principal component analysis.

Table 2 Pearson's product-moment correlations of principal component scores with schizotypy and handedness

	Total schizotypy		Absolute handedness	
	PC1	PC2	PC1	PC2
<i>Pooled</i>				
r	0.111	0.262	-0.375	-0.049
P	0.506	0.111	0.026*	0.778
<i>Females</i>				
r	0.069	-0.350	-0.469	0.0722
P	0.747	0.094	0.032*	0.756
<i>Males</i>				
r	-0.148	0.337	0.243	-0.210
P	0.613	0.239	0.403	0.470

Abbreviation: PC, principal component.

* $P<0.05$, in boldface.

differentially strong, relatively localized intercorrelation of methylation for the CpG sites between 78 and 85, such that individuals tend to differ among themselves in methylation across this entire set of sites.

PC1 scores were significantly negatively correlated with handedness in both sexes pooled ($P=0.026$; Table 2), and in females ($P=0.032$). As the PC1 loadings (Table 1) suggest that this inverse relationship between methylation level and strength of handedness was driven by variation in the CpG78–85 block, tests of schizotypy and handedness were also performed with methylation averaged across only these sites. Average methylation across the CpG78–85 block was negatively correlated with absolute handedness in females ($r=-0.456$, $P=0.019$; Supplementary Table S7), indicating that higher methylation was associated with more mixed handedness. This association was not significant for both sexes pooled ($P=0.148$) or for males ($P=0.096$; Supplementary Table S7). Tests for associations of PC1 and PC2 with schizotypy showed no significant results for males and females, either separately and pooled ($P>0.05$; Supplementary Table S7).

DISCUSSION

Schizophrenia-risk genotypes of the imprinted gene *LRRTM1* were associated with higher total schizotypy, with the effect mediated primarily by females. This result is consistent with predictions based on findings by Francks *et al.*⁷ and Ludwig *et al.*,⁸ although these studies did not test for differences between the sexes. Taken together, these studies and the results reported here support the existence of a genetically based continuum between schizophrenia and schizotypy, here mediated by a gene subject to imprinting. Imprinted genes and syndromes strongly associated with schizophrenia include *GABRB2*, a gene that modulates GABAergic neurotransmission,⁴ and Prader–Willi syndrome, a condition caused by absence of expression for a small set of paternally expressed imprinted genes on chromosome 15, which represents one of the most penetrant known causes of schizophrenia and psychosis.³⁶

Although the schizophrenia-associated haplotypes of *LRRTM1* are rare (about 1–2% in the population studied here and in HapMap Caucasian populations such as CEU), the risk alleles involved are more frequent in some other human groups (for example, for rs1446109 HapMap data: Han Chinese HCG 0.337 and Japanese JPT 0.395), suggesting the possibility of different population-wide effects in such groups. Moreover, the magnitude of the schizotypy difference between individuals with risk and non-risk genotypes in the population studied here was substantial, about three-quarters of a s.d. overall and over a s.d. for females considered separately. The relatively low population frequency and relatively highly penetrant effects of the *LRRTM1* risk haplotype indicate that it resembles a copy number variant in these respects,³⁷ although for a single small locus and gene. These considerations suggest that the *LRRTM1* risk alleles analyzed here may be useful in developing a single-locus model for high expression of schizotypal traits, which could help to clarify the nature and causes of continua between schizophrenia, schizotypy, and typical cognition.

In contrast with these results for schizotypy, no effect was found of *LRRTM1* SNP genotypes on handedness. This finding is consistent with the results of Francks *et al.*⁷ of no effects of the same SNPs in a nonclinical sample, and that of Arning *et al.*,³⁸ showing that the *LRRTM1* SNP rs6733871 (which showed significant association with handedness in the study by Ludwig *et al.*⁸ of individuals with dyslexia) also showed no association with handedness in a nonclinical population. Associations of *LRRTM1* SNPs with handedness in two studies of individuals with dyslexia,^{7,8} and the lack of such

associations in three nonclinical samples, suggest that the *LRRTM1* SNPs analyzed here influence handedness only in individuals with dyslexia. Such a restriction in genotypic effects may be related to epistatic interactions between handedness-related genes and dyslexia risk genes,^{39,40} the atypical lateralization and handedness found in dyslexia,⁴¹ or the notable neurocognitive overlaps between dyslexia and schizophrenia.^{42–44} Comparable results have been found for the gene *PCSK6*: the SNP rs11855415 in this gene showed genome-wide association with handedness among individuals with dyslexia, but no association in two large nonclinical samples.^{38–40} Instead, a variable number of tandem repeats (VNTR) polymorphism in *PCSK6*, rs10523972, showed strong association with handedness in a nonclinical population.

Higher *LRRTM1* methylation levels, involving a block of contiguous CpG sites, were found to be associated with more-mixed handedness in both sexes, with the effect mediated primarily by the data for females. By contrast, CpG methylation levels were not associated with schizotypy in any of the analyses.

Epigenetic influences on handedness have not previously been tested for, or documented, and suggest that handedness and associated cerebral asymmetries are mediated in part by environmental factors, such as fetal testosterone levels, that modify the expression of specific genes via methylation during brain development (for example, study by Hampson *et al.*⁴⁵). Higher levels of methylation in the promoter regions of genes are normally indicative of lower gene expression,^{30,31} which in this case would generate a relative maternal bias because *LRRTM1* is paternally expressed (maternally silenced), although this hypothesis requires empirical testing.

Why and how should schizophrenia, schizotypy, and handedness be connected with genomic imprinting, according to the well-supported kinship theory of imprinting? Two nonexclusive hypotheses, both supported by some previous work, may explain these results. First, the development of schizophrenia and psychosis is notably associated with insecure psychological attachment to the mother, with Prader–Willi syndrome representing an apparent extreme example of this phenomenon.⁴⁶ To the extent that insecure attachment involves reduced demands imposed on the mother, its expression may be mediated in part by maternally biased expression of imprinted genes, according to the kinship theory.¹ Second, some aspects of schizotypy may be associated with enhanced empathic cognition,^{47–49} which could tend to favor matrilineal interests.^{1,49,50} This hypothesis is supported by links of the imprinted schizophrenia-risk gene *GABRB2* with altruism in humans,⁴ but requires further tests.

Most generally, these results support the hypothesis that schizophrenia, schizotypy, and handedness are mediated in part by effects from genomic imprinting. Such findings should motivate the genetic and epigenetic analysis of additional brain-imprinted genes with regard to their effects in mediating human cognition, laterality, and disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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