

ORIGINAL ARTICLE

Expanding the range of *ZNF804A* variants conferring risk of psychosis

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Received 29 July 2009; revised 1 October 2009; accepted 21 October 2009; published online 5 January 2010

A trio of genome-wide association studies recently reported sequence variants at three loci to be significantly associated with schizophrenia. No sequence polymorphism had been unequivocally ($P < 5 \times 10^{-8}$) associated with schizophrenia earlier. However, one variant, rs1344706[T], had come very close. This polymorphism, located in an intron of *ZNF804A*, was reported to associate with schizophrenia with a P -value of 1.6×10^{-7} , and with psychosis (schizophrenia plus bipolar disorder) with a P -value of 1.0×10^{-8} . In this study, using 5164 schizophrenia cases and 20709 controls, we replicated the association with schizophrenia (odds ratio OR = 1.08, $P = 0.0029$) and, by adding bipolar disorder patients, we also confirmed the association with psychosis (added $N = 609$, OR = 1.09, $P = 0.00065$). Furthermore, as it has been proposed that variants such as rs1344706[T]—common and with low relative risk—may also serve to identify regions harboring less common, higher-risk susceptibility alleles, we searched *ZNF804A* for large copy number variants (CNVs) in 4235 psychosis patients, 1173 patients with other psychiatric disorders and 39481 controls. We identified two CNVs including at least part of *ZNF804A* in psychosis patients and no *ZNF804A* CNVs in controls ($P = 0.013$ for association with psychosis). In addition, we found a *ZNF804A* CNV in an anxiety patient ($P = 0.0016$ for association with the larger set of psychiatric disorders).

Molecular Psychiatry (2011) 16, 59–66; doi:10.1038/mp.2009.149; published online 5 January 2010

Keywords: schizophrenia; bipolar disorder; *ZNF804A*; CNV; association

Introduction

Before the publication of recent genome-wide association (GWA) studies,^{1–3} the sequence variant having the strongest evidence for unconditional association with schizophrenia was rs1344706[T]. This variant was reported to be associated with schizophrenia with an odds ratio (OR) of 1.12 and a P -value of 1.6×10^{-7} . Evidence for association was strengthened (OR = 1.12, $P = 1.0 \times 10^{-8}$) when a psychosis phenotype (schizophrenia plus bipolar disorder) was assessed.⁴ More recently, rs1344706 was shown to be associated with alterations in the functional connectivity of various regions of the brain.⁵ The single-nucleotide polymorphism (SNP) is located in an intron of *ZNF804A*, a gene encoding a protein predicted to be a transcription factor.

Current discussions of GWA studies have suggested that findings of common SNP association may be exploited by examining the region surrounding the initial variant for additional polymorphisms not tagged by the original variant.^{6–8} Of particular interest are low frequency (between 5 and 1%) and rare (<1%) susceptibility polymorphisms that may confer a higher risk than the originally described variant but are unlikely, because of their frequency, to be discovered by standard GWA study. An example of the successful use of this strategy is in nonalcoholic fatty liver disease where the finding of a common *PNPLA3* susceptibility allele was followed by the discovery, using re-sequencing, of an excess of null sequence mutations in individuals with the highest hepatic fat levels as well as a protective allele that was rare in European Americans (about 0.3%) but common in African Americans (about 10%).⁹ To our knowledge, the discovery of rare copy number variants (CNVs) associated with disease in regions initially uncovered through common SNP susceptibility alleles has not been reported.

In this study, we confirmed the association of rs1344706[T] with schizophrenia and also corroborated the bolstering of the evidence when the

phenotype was expanded to psychosis. In addition, we examined *ZNF804A* for large structural variants, and found CNVs in three patients with psychiatric disorders, but not in controls.

Materials and methods

Samples

Full information about each study group is presented in the Supplementary Methods. Individuals were diagnosed according to International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) or Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria. Because of variation in diagnostic criteria and protocols between the various centers, some phenotypic heterogeneity is expected. All individuals provided written, informed consent for participation and approval was obtained from the ethics committees at each location.

In the initial analysis of rs1344706 association with schizophrenia, genome-wide typed samples from seven European locations were included. After quality control, case/control samples from England (93/88), Finland/excluding Kuusamo (59/147), Finland/Kuusamo (123/50), Iceland (589/11492), Italy (84/89), the Netherlands (693/3689) and Scotland (658/661) were included. With the exception of the samples from the Netherlands, these samples derived from the genome-wide data described in our primary GWA report.³ Additional samples from Bonn and Munich that were part of the earlier data set were not used here as they had been included in the initial *ZNF804A* study.⁴ Bipolar samples from Iceland ($N = 404$ after quality control) were also incorporated into the psychosis analysis.

In the follow-up study of rs1344706 association with schizophrenia, samples from nine locations (after quality control)—China (460 cases, 466 controls), Denmark/Aarhus (236 cases, 500 controls), Denmark/Copenhagen (513 cases, 1338 controls), Germany/Bonn (275 cases, 510 controls), Germany/

Munich (178 cases, 320 controls), Hungary (264 cases, 223 controls), Norway (201 cases, 357 controls), Russia (483 cases, 487 controls) and Sweden (255 cases, 292 controls)—were included. The European samples in this set originated from ‘follow-up set 2’ of our primary GWA study,³ although some samples that were used in the original *ZNF804A* study⁴ or that lacked sufficient DNA for typing were excluded. In addition, 205 bipolar samples from Norway were part of the psychosis analysis.

The CNV portion of the study included schizophrenia case/control samples from 10 European locations (following quality control): Denmark (547/541), England (85/80), Finland/excluding Kuusamo (61/144), Finland/Kuusamo (129/49), Germany/Bonn (485/848), Germany/Munich (585/586), Iceland (603/35 995), Italy (84/86), the Netherlands (604/489) and Scotland (645/663). These samples corresponded to the genome-wide typed samples used in our initial SNP association study,³ supplemented by material from Denmark and the Netherlands. Finally, 407 bipolar and 1173 depression, anxiety and anxiety-related disorder samples from Iceland were examined.

Genotyping

Genome-wide genotyping for all samples from England, Finland, Iceland and Italy was carried out at deCODE Genetics using the Illumina HumanHap300 chip (San Diego, CA). For the samples from Germany/Munich and Scotland, approximately one-third of both cases and controls were genome-wide typed at deCODE using the Illumina HumanHap300 chip, whereas the remaining two-thirds of cases and controls were genome-wide typed at Duke University using the Illumina HumanHap300 (Germany) and Illumina HumanHap550 (Scotland) chips. For the samples from the Netherlands, 715 cases and 643 controls were typed at UCLA using the Illumina HumanHap550 chip, whereas 3334 additional controls were typed at deCODE using the Illumina HumanCNV370 chip. The samples from Germany/Bonn were genome-wide typed at the University of Bonn using the Illumina HumanHap550 chip. The samples from Denmark were typed at deCODE Genetics using the Illumina HumanHap610 chip. For all the chips, yield for the two markers used for the surrogate (rs12477914 and rs1366840) was at least 98% and, in the controls of each group, neither marker deviated significantly from Hardy–Weinberg equilibrium ($P > 0.05$). For both the markers used, when the same study group was typed on more than one chip, there was no significant difference in allele frequency between the chips in either cases or controls ($P > 0.05$).

Of the follow-up groups, the Norwegian sample was genotyped at the University of Oslo using the Affymetrix 6.0 chip (Santa Clara, CA, USA). The remaining groups were single-marker genotyped at deCODE Genetics using Centaurus assays (Nanogen, San Diego, CA, USA). Yield for rs1344706 was at least

94% in both cases and controls of all study groups and, in the controls of each group, rs1344706 did not deviate significantly from the Hardy–Weinberg equilibrium ($P > 0.01$).

Sample quality control

For the genome-wide typed samples, duplicate samples, samples with a sex determined by X-chromosome homozygosity different from their reported sex and samples determined to be of non-European ancestry either by running STRUCTURE¹⁰ using the HapMap CEU, YRI and CHB/JPT individuals as reference samples or by examination of identity by state were removed. In addition, low-yield (<98% for the Illumina chips and <95% for the Affymetrix chip) samples were removed for the SNP association part of the study, whereas samples with an excess of large CNVs (>10 CNVs of at least 10 SNPs) were removed for the CNV part of the study. For the single-marker typed samples, samples that, based on additional genotyping, were duplicates of other samples in the data set or were low yield (<60%) were removed.

Association analysis

Association analysis of rs1344706 was carried out using a likelihood procedure described previously.¹¹ For the genome-wide typed samples, a surrogate for rs1344706 made up of a linear combination of two-marker haplotypes, defined using the HapMap CEU, was used. This method, which we have used earlier,¹² is an extension of the two-marker haplotype tagging described in Pe'er *et al.*¹³ and is similar in spirit to the methods described in Nicolae¹⁴ and Zaitlen *et al.*¹⁵ Genomic control¹⁶ was used to correct for relatedness and potential population stratification in each genome-wide typed study group. With the exception of Iceland, genomic control factors were <1.1; in Iceland, some related individuals were included in the analysis and genomic control factors were 1.19, 1.12 and 1.18 for the schizophrenia, bipolar and psychosis analyses, respectively. The study groups within the Illumina genome-wide typed and follow-up sets were combined using the Mantel–Haenszel model.¹⁷ The combined Illumina genome-wide typed and combined follow-up sets were joined using summary statistics. *P*-values were calculated by summing *z*-scores with each data set's *z*-score multiplied by the inverse of that data set's s.e. divided by the square root of the sum of the squared inverse s.e., and combined ORs were calculated by summing log ORs with each log OR weighted by the inverse of its variance. The CNV association analysis was carried out using exact Mantel–Haenszel tests.

CNV detection

PennCNV,¹⁸ a free, open-source tool, was used for copy number variation detection. The input data for the program are log R ratio, a normalized measure of the total signal intensity for the two alleles of the SNP, and B-allele frequency, a normalized measure of the

allelic intensity ratio of the two alleles. Values of these quantities are derived with the help of control genotype clusters (HapMap samples), using Illumina BeadStudio software. A hidden Markov model is then used to make CNV calls based on the probability of a given copy state at the current marker as well as the probability of observing a copy state change from the previous marker to the current one. A built-in correction model for GC content¹⁹ is included. As the samples were genotyped on several different types of Illumina BeadArray chips (HumanHap300, HumanHap300-duo, HumanHap550, HumanHap610), we analyzed them with a twofold approach: first, using the full complement of markers on the chip and second, using only a subset of markers, present on most of the chip types, to ensure similar resolution of markers covering the genome. In this study, only CNVs including at least ten consecutive markers in the region chr2:185,171,338–185,512,459 Mb NCBI Build 36 were considered.

Results

In an attempt to replicate the association of rs1344706[T] with schizophrenia, we used two data sets: (1) an initial set of Illumina genome-wide typed samples from Europe nonoverlapping with the samples of the original report⁴ (2299 cases, 16 216 controls) and (2) a set of follow-up samples from Europe and China also without an overlap with those of the initial study⁴ (2865 cases, 4493 controls). Because rs1344706 was not included on the Illumina HumanHap300 or

HumanHap550 BeadChips, a surrogate, composed of a linear combination of haplotypes, was used in the analysis of the genome-wide data.

The OR for rs1344706[T] (or the surrogate) was 1.09 in the initial data set and 1.08 in the follow-up data set; *P*-values were 0.037 in the initial sample and 0.033 in the follow-up sample (Table 1). In the combination of the two data sets, the OR was 1.08 and the *P*-value was 0.0029 (Table 1). The average control frequency in the combined sample was 0.59, identical to that of the original report.⁴ We found no evidence of heterogeneity between the study groups (*P*=0.66, Supplementary Table 1) and no indication that we should reject the multiplicative model for the full model (*P*=0.95 for the initial sample, *P*=0.79 for the follow-up sample).

We also examined the association of rs1344706[T] with psychosis by including bipolar samples from Iceland (*N*=404) and from Norway (*N*=205). The strength of the evidence for association increased (*P*=0.00065 from *P*=0.0029, see Table 2 for psychosis analysis and Supplementary Table 2 for bipolar-only analysis), consistent with the results of the initial report.⁴

To explore the suggestion that loci initially identified through GWA studies may also harbor independent susceptibility variants, perhaps rarer and of greater effect, we examined the region overlapping *ZNF804A* (chr2:185,171,338–185,512,459 in NCBI Build 36) for large (containing at least 10 consecutive SNPs) CNVs in 3828 schizophrenia patients, 407 bipolar disorder patients and 39 481 controls not

Table 1 Association of rs1344706[T] with schizophrenia

Data set (N cases/N controls)	Control frequency	OR (95% CI)	<i>P</i> -value
Initial set (2280/16 099)	0.58	1.09 (1.01, 1.18)	0.037
Follow-up (2797/4407)	0.59	1.08 (1.01, 1.16)	0.033
Initial set and follow-up (5077/20 506)	0.59	1.08 (1.03, 1.14)	0.0029

Abbreviations: CI, confidence interval; OR, odds ratio.

Control frequency is the average, unweighted allele frequency over the various study groups of each data set, whereas ORs and *P*-values were estimated using the Mantel–Haenszel model. Analysis for each genome-wide typed group was adjusted using genomic control (see Materials and methods).

Table 2 Association of rs1344706[T] with psychosis

Data set (N cases/N controls)	Control frequency	OR (95% CI)	<i>P</i> -value
Initial set (2683/16 099)	0.58	1.10 (1.02, 1.18)	0.012
Follow-up (2991/4407)	0.59	1.08 (1.01, 1.16)	0.020
Initial set and follow-up (5674/20 506)	0.59	1.09 (1.04, 1.14)	0.00065

Abbreviations: CI, confidence interval; OR, odds ratio.

Control frequency is the average, unweighted allele frequency over the various study groups of each data set, whereas ORs and *P*-values were estimated using the Mantel–Haenszel model. Analysis for each genome-wide typed group was adjusted using genomic control (see Materials and methods).

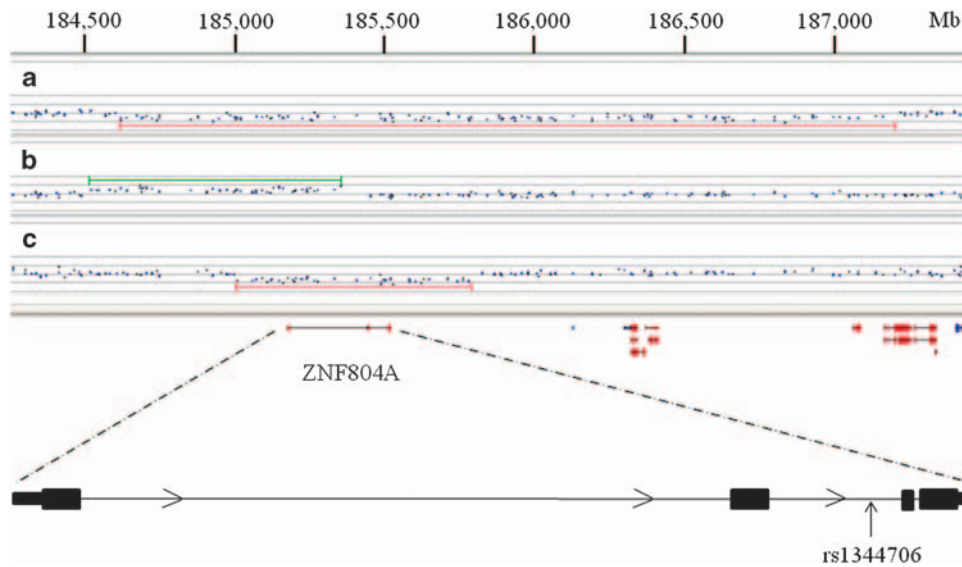


Figure 1 Large structural variants in *ZNF804A*. (a) A deletion in an Icelandic anxiety patient (patient 3), (b) a duplication in an Icelandic bipolar patient (patient 2), (c) a deletion in a Scottish schizophrenia patient (patient 1). The coordinates are based on NCBI Build 36.

known to have any psychiatric disorder (see Materials and methods). In addition, 1173 patients with other psychiatric disorders (945 anxiety or anxiety-related disorder patients and 662 depression cases, 434 of whom were also diagnosed with anxiety or anxiety-related disorders) were available.

We carried out tests for association with schizophrenia and psychosis and also with the broader phenotype of combined psychiatric disorders. A schizophrenia patient harbored a deletion of the entire *ZNF804A* gene (Figure 1), but no *ZNF804A* structural variants were identified in the control set, leading to a P -value of 0.49 for association with schizophrenia. A bipolar disorder patient had a duplication of the first exon of the gene (Figure 1), resulting in a P -value of 0.013 for association with psychosis. A *ZNF804A* deletion was also identified in a patient with anxiety (Figure 1), leading to a P -value of 0.0016 for association with the larger set of psychiatric disorders.

Carriers of *ZNF804A* CNVs were of both sexes and were generally unremarkable with respect to age of onset and other features of their disorder (Supplementary Table 3). For the duplication carrier, genotypes from the patient's father and from a sibling of the patient's mother were available. Because the mother's sibling carried the haplotype background the duplication was located on, but not the duplication, it could be inferred that the duplication event had most likely taken place either in the germ line of the patient's mother or during gametogenesis in one of the maternal grandparents. For the deletion carriers, genotypes were not available for either the parents or the parents' relatives; thus, it was not possible to estimate the timing of the mutation.

Discussion

In the first part of this study, we confirmed the association of rs1344706[T] with schizophrenia ($P=0.0029$), using genotypes from 5164 schizophrenia cases and 20 709 controls. Although, in this study, only a modest number of bipolar disorder cases was available ($N=609$), adding these cases to the analysis increased the evidence for association, in line with the original report. Thus, rs1344706[T] may confer risk of both schizophrenia and bipolar disorder, a finding consistent with epidemiological studies suggesting shared genetic risk factors for the two diseases.^{20,21}

Meta-analysis P -values for rs1344706[T], based on the results from this study and the original report, were 2.8×10^{-9} (OR = 1.10) for schizophrenia and 3.8×10^{-11} (OR = 1.11) for psychosis, making association with both of these phenotypes unequivocal. In addition, three schizophrenia GWA studies^{1,2,22} recently reported association results for rs1344706[T], although the samples used by each study at least partially overlap the material included here or in the original study. The International Schizophrenia Consortium (ISC) achieved a one-tailed P -value of 0.029 (OR = 1.08) based on 2519 cases and 2110 controls, of which about 650 cases and 650 controls were included in this study.¹ The Molecular Genetics of Schizophrenia (MGS) consortium reported a two-tailed P -value of 0.026 (OR = 1.10) using about 5300 samples, of which ~1900 were part of the original *ZNF804A* study.² A third GWA study gave results for a rs1344706 surrogate;²² however, the Aberdeen samples used in that study are entirely incorporated into this study. In addition, while this work was under review, a

replication study using the Irish Case–Control Study of Schizophrenia (ICSS) sample reported a one-tailed *P*-value of 0.011 (OR = 1.20) based on a unique group of 993 cases and 570 controls.²³ Taken together, the ISC, MGS and ICSS reports are consistent with an association between rs1344706[T] and schizophrenia, and they also provide additional support for that conclusion.

In the second part of the study reported in this paper, we examined the *ZNF804A* region for gain or loss of copy number, using 4235 psychosis patients, 1173 patients with other psychiatric disorders and 39481 controls. Two psychosis patients harbored CNVs affecting at least part of *ZNF804A*, resulting in a *P*-value of 0.013 for association with psychosis. In addition, a patient with anxiety had a deletion, leading to a *P*-value of 0.0016 for association with psychiatric disorders.

The CNV portion of this study involved a large data set, but additional information about CNVs is also publicly available. In approximately 12 000 controls from studies included in the Database of Genomic Variants²⁴ and three other reports,^{25–27} two CNVs involving *ZNF804A* exons, both from the same study,²⁸ are found. One event is a deletion of the entire gene in an African-American parent–offspring pair, and the second is a deletion affecting the 3' exons of the gene in a European-American child. Although these CNV carriers are classified as healthy controls, the children, aged 0–18 years, may subsequently develop psychiatric disorders, and the parent, though free of major medical problems, does not seem to have been extensively interviewed for psychiatric disorders. In about 3800 schizophrenia cases not overlapping with those in this report^{25,27,29,30} and about 1000 bipolar cases,²⁶ no additional *ZNF804A* events are found (note that the *ZNF804A* deletion observed in the ISC data set¹ is from an individual also included in this study), but in approximately 1700 autism families and 400 unrelated autism cases,^{31–36} there is a duplication of the entire gene in two affected siblings and a partial *ZNF804A* duplication in another patient. Because of the incompleteness of the available phenotypic information, drawing strong conclusions about the association of *ZNF804A* CNVs and mental disorders from the publicly accessible data is difficult. However, considered together with the association between *ZNF804A* CNVs and psychiatric disorders observed in this report, the existence of two *ZNF804A* deletion events in controls, several young, and all without detailed phenotype information, and the presence of two *ZNF804A* duplication events in autism patients, suggests that the link between *ZNF804A* CNVs and mental disorders should be investigated further.

This is, to our knowledge, the first report of the identification of rare, disease-associated CNVs in a region initially discovered through common SNP association. Genes implicated in Mendelian disorders, however, have frequently been found to display

a broad spectrum of mutations. In the cystic fibrosis gene, *CFTR*, more than 1600 mutations have been identified (Cystic Fibrosis Mutation Database; <http://www.genet.sickkids.on.ca/cftr/app>), including large structural events. Rare, risk-conferring sequence mutations have also been identified in genes such as *PNPLA3* that were initially uncovered through common SNP association.⁹ Similarly, some genes such as *TCF2* first connected to disease through rare, highly penetrant risk alleles were later found, through GWA studies, to harbor common susceptibility alleles of modest effect.³⁷ At *CNTNAP2*, the earliest reports associated rare SNPs with epilepsy and autism;³⁸ more recently, evidence of the association of common SNPs with autism, although not at the genome-wide significant level, has been described,^{39,40} and rare structural variants in individuals with autism have also been identified.^{41–43}

Association with disease is more difficult to establish for very rare genetic variants. In this study, we pooled deletions and duplications, considering them together. This approach allowed us to present statistical evidence for the connection of *ZNF804A* CNVs to psychiatric disorders. In addition, the large-scale nature of the structural alterations reported here makes phenotypic effects more likely, compared with most sequence changes. Both the deletions identified here remove the entire sequence of *ZNF804A* (Figure 1), which is likely to result in differences in mRNA abundance that may have downstream consequences, especially because *ZNF804A* is a putative transcription factor, a class of protein that has often been implicated in haploinsufficiency disorders.⁴⁴ The duplication that includes one or two exons of *ZNF804A* (Figure 1) may lead to a protein that acts in a dominant-negative manner, interfering with the actions of the wild-type *ZNF804A*, or, because of the manner in which the duplicated sequence is inserted, the event may result in alterations in the transcription of the original copy of *ZNF804A*.

Mouse models may be useful in helping to understand the functional consequences of these variants. The reverse may also be true: these rare structural variants may turn out to be more valuable than the originally identified common variant in establishing a link between *ZNF804A* and a psychosis-related phenotype in an animal model. This is because gene knockout or overexpressing mice can be engineered relatively easily, the penetrance of pathogenic CNVs is generally much higher than common SNP variants, and temporal or cell specific manipulation of expression can be carried out with conditional mutants.

The *ZNF804A* variants examined in this study confer risk of more than one category of disease. Rs1344706[T] is associated with both schizophrenia and bipolar disorder, and the CNVs identified here suggest a connection with anxiety as well, although we do not find evidence for the association of rs1344706[T] with anxiety (data not shown). The functional connectivity alterations identified in the

brains of rs1344706[T] carriers⁵ provide a possible mechanism for the link between *ZNF804A* variants and a diversity of disorders. Thus, examination of brain functional connectivity in *ZNF804A* CNV carriers would be of interest.

In this study, we have replicated the previously reported association of rs1344706[T] with schizophrenia and psychosis. In addition, we have further explored the locus and have identified rare CNVs in patients with psychiatric disorders, but not in controls.

Conflict of interest

Some of the authors, including Kari Stefansson (CEO of deCODE Genetics) and Augustine Kong (VP Statistics of deCODE Genetics), are shareholders in deCODE Genetics.

Acknowledgments

We thank the subjects, their families and the recruitment center staff. This work was supported by the European Union (LSHM-CT-2006-037761 (Project SGENE), PIAP-GA-2008-218251 (Project PsychGene) and HEALTH-F2-2009-223423 (Project PsychCNVs)), the National Genomic Network (NGFN-2) of the German Federal Ministry of Education and Research (BMBF), the National Institute of Mental Health (R01 MH078075), the Center of Excellence for Complex Disease Genetics of the Academy of Finland (Grants 213506, 129680) and the Biocentrum Helsinki Foundation and Research Program for Molecular Medicine, Faculty of Medicine, University of Helsinki.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)

Appendix

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