The Genetics of Familial Psychiatric Disorders: Insights into Genes and Mechanisms

Christian Gerhard Bouwkamp

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The Genetics of Familial Psychiatric Disorders: Insights into Genes and Mechanisms

De genetica van familiaire psychiatrische stoornissen: Inzicht in genen en mechanismen

Thesis

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Christian Gerhard Bouwkamp
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Erasmus University Rotterdam

Doctoral Committee

Promotors:	Prof.dr. S.A. Kushner Prof.dr. V. Bonifati
Other members:	Prof.dr. Y. Elgersma Prof.dr. H.W. Tiemeier Dr. P.M.J. Haffmans

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Introduction and scope

Introduction and scope

Psychiatric disorders are common in the population and historical as well as cultural evidence demonstrates their presence throughout history.[1,2] Also psychiatric hospitals offering treatment for the mentally ill have been around since the medieval period in the Arab world.[3] The first time the word *psychiatry* was used however was in a publication by the German professor Johan Christian Reil at Halle University in 1808. He saw psychiatry as a core medical discipline next to surgery and general medicine which was to be practiced only by the best physicians.[2] It is since this time that we speak of modern psychiatry. In his or her life, about 20 percent of the population will suffer from a psychiatric disorder.[4] The life-time prevalence of the hallmark severe psychiatric disorders schizophrenia, bipolar disorder and autism spectrum disorders (ASD) is rougly five percent.[5,6] The best indicator for disease risk is a close relative with a psychiatric disorder.[7]

The focus of the field in the 19th century as well as for much of the 20th century was on the description and epidemiology of psychiatric disorders. Major contributors of the 19th century in this regard are German psychiatrist Emil Kraepelin and Swiss psychiatrist Eugen Bleuler. Kraepelin devised the term dementia praecox which was later described as the *Group of Schizophrenias* by Bleuler.[8] Kraepelin devised a systematic classification of psychiatric illness based on the clinical symptomatology. This is often considered the basis for the current classification systems, the *Diagnostic and Statistical Manual of Mental disorders*, now in its fifth edition (DSM-V) and the *International Classification of Disease*, now in its tenth edition (ICD-10). In these classification systems, no relationship ought to be assumed with respect to the underlying aetiology of the psychiatric disorders. These current classification systems are based on the clinical phenomenology and to a large degree are not based on the biological aetiology of disease.

As a consequence, in the current classification systems there is considerable overlap between disease categories, such as schizophrenia and bipolar disorder[9], as well as personality disorders.[10] In addition, there is considerable heterogeneity within disease categories due to the systems' requirements to meet at least a minimum number of symptoms out of a larger number of possible symptoms.[7] Mathematically, this leads to many possible combinations of syndromes with the same classification.

Early genetic studies in psychiatry

Kraepelin already described the increased prevalence of psychiatric disorders in family members of patients. Since the publication of the work on the crossing of pea plants by Gregor Mendel, on what is now known as Mendelian Genetics, statistical genetic and epidemiological studies were performed in groups of patients with psychiatric disorders.[11] In these early stages, it was found that the concordance rate for schizophrenia of monozygotic twins was ~50 percent. These numbers are similar to the ones found back in the more recent twin studies on psychiatric disorders.[12] Through these large epidemiological studies, it has been possible to calculate the recurrence rates in, for example, schizophrenia.[13]

Candidate gene approach

The major biological and technological developments in the second half of the 20th century significantly boosted the studies attempting to unravel the genetic and neurobiological underpinnings of psychiatric disorders. Through the working mechanism of psychotropic medication and other hypothesized biological deficiencies thought to underlie mental illness, many genes have been proposed to be implicated in the aetiology of psychiatric disorders.[14] These candidate genes, implicated in processes such as neurodevelopment, dopamine signalling or glutamate receptor signalling, as well as cell-to-cell communication and neuronal plasticity, were screened by Sanger sequencing technology in order to determine the mutational burden in cases compared to controls. The rationale that these genes and their encoded products would be involved has historically been based on their role in the biological systems hypothesized to be involved in the aetiology of the disorder, such as the glutamate receptor and dopaminergic signalling pathways in schizophrenia[15] and the biological clock genes[16–19] and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in bipolar disorder.[20] What would nowadays be considered small cohorts of cases and controls were screened with mixed positive and negative results. The historical candidates for schizophrenia have recently been reviewed by expert-geneticists[14] and additionally, the genome-wide association (GWAS) data from the psychiatric genomics consortium (PGC) do not implicate most of the historical candidate genes as major risk factors for schizophrenia. Exceptions are several genes associated with glutamatergic signalling and the gene *DRD2* which codes for the dopamine 2 receptor, the mechanism of action of antipsychotic medication.[21] The argument for the historically difficult-to-obtain genetic signal is the bias towards the *a priori* biological function

of the candidate genes. Consequently, there is little sound genetic evidence of involvement of these candidate-genes in the aetiology of psychiatric disorders.

Mendelian Genetics

Given the clustering of psychiatric disorders in families, many family-based studies have been performed. In Mendelian genetics, linkage analysis is used in order to identify chromosomal *loci* in a pedigree which are 'linked' to traits or conditions. There were two major papers published in the late 1980s on bipolar disorder[22] and schizophrenia[23] showing statistical significant linkage to chromosome 11 and 5 respectively. After that, many researchers attempted family-based linkage analyses, which resulted in many loci found to be significantly linked with psychiatric illness, but only few of them replicated. A meta-analysis of family-based linkage studies in 3255 families with schizophrenia comprising 7413 cases was published describing that only suggestive linkage was found on the long arm of chromosome five and the long arm of chromosome two. In Europeans specifically, the authors found suggestive linkage on the short arm of chromosome eight. The authors state however that their analysis showed many more loci might be linked to schizophrenia.[24] With schizophrenia as hallmark psychiatric disorder, these findings illustrated the complicated genetic architecture of the major psychiatric disorders.[25]

Currently in many fields of medicine, clinicians and researchers apply linkage analysis coupled with exome sequencing, which allows the reading of the protein-coding fraction of the DNA, in single patients and in families with phenotypes of interest.[26,27] This is done in search of segregating Mendelian variants that confer a strongly increased risk of illness. The search for Mendelian variants underlying human disease continues with great success. Increasingly more variants are being identified underlying phenotypes which were previously difficult to diagnose.[28] The NIH-funded initiative *Centers for Mendelian Genomics (CMG)* has identified 956 genes of which 375 were not yet associated with human health and disease in the past five years. These data demonstrate Mendelian genetics is a vivid field of study. Additionally, high-penetrance coding variants provide us with the unique opportunity to study its consequence in a laboratory environment. For variants which are in the coding regions of a gene, the functional consequences are relatively well understood and can be modelled in a laboratory setting in cell culture or model organisms. Given the complex genetic architecture underlying psychiatric disorders indicating a polygenic aetiology, it appears a common major genetic

vulnerability factor for psychiatric disorders does not exist. The new generation of Mendelian genetics whereby classical family linkage studies are combined with the next generation sequencing (NGS) technologies has the potential to unravel the genetic architecture in a family-per-family fashion. In the era of genome-wide association studies in psychiatry, the field of Mendelian genetics studying families with psychiatric phenotypes has been neglected. However, from a relative-risk-point-of-view, as well as regarding the possibilities of studying the aberrant biology in a laboratory situation, Mendelian forms of psychiatric illness provide an excellent opportunity to gain insight into the disease pathophysiology because of the rare variants with a large effect size. As is the case for the major neurological disorders Alzheimer's disease, Parkinson's disease and fronto-temporal dementia (FTD), our current medical-biological knowledge with respect to the pathophysiology of these disorders is derived from families with Mendelian pathogenic mutations.

Genome wide association studies (GWAS)

The development of massive parallel genotyping assays in the form of microarray technology allowing for simultaneous enquiry of hundreds of thousands of single nucleotide polymorphisms (SNPs) led to setting up large consortia studying complex traits and disease phenotypes such as psychiatric disorders. This methodology allows for statistical association studies of many genetic markers (the SNPs) which are common in the population with membership of the patient group versus the control group, or alternatively they can be associated to a continuous trait such as height. Given the vast number of statistical test performed, multiple testing correction has to be applied which leads to very stringent criteria of statistical significance. The most recent iterations of GWAS studies for the psychiatric disorders are led by the Psychiatric Genomics Consortium (PGC). Regarding schizophrenia, a GWAS was performed with over 30,000 patients and more than 120,000 controls. This has resulted in 108 significantly associated independent loci containing variants with relatively low effect sizes.[21] Additionally, a large case-control association study was performed using exome sequencing data instead of SNP array data. This study was performed on ~2500 cases and ~2500 controls from Sweden. The authors of the study however did not identify variants that met genome-wide significance after multiple testing correction[29], indicating that also a technology which allows for higher resolution enquiry of the genomic DNA does not per se allow for detection of disease-relevant variants. Recently, a strong association signal found on chromosome 6 by GWAS studies for schizophrenia, partially due to variations of the complement component 4 (C4) which were associated with brain expression levels of C4 and

complement component 5 (C5). The schizophrenia-associated variation was associated with higher C4 expression levels. It has been shown that C4 has a role in synaptic pruning and therefore could explain part of the pathology in schizophrenia.[30] For bipolar disorder, a GWAS has been performed with ~12,000 patients and ~52,000 controls. In this study, significant association was found for markers close to *CACNA1C and ODZ4*.[31] For major depressive disorder (MDD), no significant SNPs had been identified in the 2013 GWAS comprising ~9,200 cases and ~9,500 controls in the discovery phase and ~6,800 cases and ~50,000 controls in the replication phase.[32] However recently 15 genetic loci have been published to be associated with the risk of MDD in a study that looked at ~75,000 patients with self-reported MDD and ~231,000 controls. Suggestively associated SNPs were brought forward to a replication phase with ~45,000 self-reported MDD patients and ~106,000 controls. The polygenic risk score was also associated with secondary phenotypes such as taking antidepressant medication, medication for mental health and anxiety.[33] For autism, it is well known that many rare monogenic syndromes exist that overlap with autism spectrum disorders (such as Fragile X syndrome and Tuberous Sclerosis Complex). Recent genetic studies investigating idiopathic autism spectrum disorder have been performed by the Simons Foundation and these studies have focused on identifying *de novo* mutations and are thus not present in the parents of the proband. A large recent study involving 2500 families found that if *de novo* mutations are combined with copy number variants (CNVs), *de novo* coding mutations explain 30% of the variance in the simplex cases.[34]

Polygenic inheritance

Historically, human molecular genetics has focused on traits and disorders with an apparent Mendelian pattern of inheritance. To date however, the field of Mendelian genetics cannot provide a molecular mechanism for most complex traits and disorders which are common in the population.[35] It has been proposed that for disorders such as schizophrenia, a polygenic risk score would better describe the underlying genetic architecture.[21,36,37] In a study using Irish families with a high incidence of schizophrenia, it was observed that the polygenic risk score was significantly elevated in both affected as well as unaffected family members.[38] The Psychiatric Genomics Consortium recently published the largest GWAS study to date identifying 108 significantly associated loci with schizophrenia. Polygenic risk score profiling using these loci explained 3.4% of the variance. Depending on the dataset used (population-based, hospitalization-based, or ascertained for genetic studies), the decile with the highest burden of risk alleles has an odds ratio for affected status of 7.8, 15.0, and 20.3 respectively.[21]

Currently, the polygenic risk scores for individual patients however are not considered suitable for use in clinical and diagnostic settings because of lack of predictive power for an individual patient.[39,40]

Syndromic mental illness

The strongest known molecular risk indicator for syndromic psychiatric illness is carriership of the 22q11.2 microdeletion.[41] Carriers of this microdeletion have a thirty-fold increased chance to develop schizophrenia. There is however debate whether or not the clinical schizophrenia that results from this syndromic context is comparable to idiopathic schizophrenia.[42] It is well known that primary mitochondrial[43] and other genetic syndromes (such as Prader-Willi and Fragile X syndrome[44]) can present with psychiatric symptoms. Although perhaps psychiatric illness in the context of other phenotypes might be different than idiopathic mental illness, we might be able to study these cases and derive from them a theory for neurobiological underpinnings of psychiatric symptoms in general.

Copy number variants

Copy number variants (CNVs) are deletions or duplications of large physical blocks of DNA.[45,46] Consequently, their presence can significantly alter gene expression and therefore has the potential to be pathogenic. Nevertheless, CNVs frequently occur in the population with an average of 1.44 CNVs per person with a tendency for smaller CNVs to be more common. The average size in the population is ~205kb.[47] CNVs have been associated with psychiatric disorders, most prominently with schizophrenia[48] and ASD.[49,50] Studying the function of the genes comprised by the CNVs has the potential to further elucidate the aetiology of psychiatric disorders.

Comparison to the field of neurology

In the field of neurodegenerative disorders, where the understanding of the disease genetics is more advanced than in psychiatry, it is widely acknowledged there are rare, usually monogenic, forms of disease as well as idiopathic forms. Although the vast majority of Alzheimer's and Parkinson's disease cases are supposedly sporadic with significant non-Mendelian genetic contributions, roughly 5-15% of cases are familial and in a smaller fraction the causative genetic mutations could be identified. Most of our current understanding about pathological mechanisms underlying these diseases is driven by models based on these rare, familial forms. Remarkably, in most cases, the clinical phenotype does not distinguish rare monogenic forms

from sporadic forms.[26,27,51–55] Since the nature of the neurodegenerative disorders is similar to psychiatric disorders: involving the brain, of pluriform aetiology, and the concept that the diagnosis is most often made on the basis of clinical evaluation, we attempted to implement a methodology often used in neurology, consisting of a family-based genetic analysis involving parametric linkage and next generation sequencing followed by either the identification of other families with segregating variants in the candidate gene and/or determining a statistical enrichment of variants in the candidate gene in cases versus controls.[26] Recently, this type of linkage-based next generation sequencing has been proposed the method of choice because of its power in identifying pathogenic mutations or risk factors in families.[27]

Scope of this work

The concept that psychiatric disorders are heritable is broadly accepted in the field of psychiatry. It has been show that the best indicator for increased disease risk is the genetic distance to an affected relative. In this work we describe genetic studies investigating adult psychiatric as well as neurodevelopmental disorders. In addition, we have studied both non-syndromic as well syndromic patients with mental illness.

In the first section, we studied two classic adult-onset psychiatric disorders: schizophrenia and bipolar disorder.

In **chapter two**, we describe a Dutch family with a high incidence of the hallmark adult-onset psychiatric disorder schizophrenia. Through linkage analysis coupled with exome sequencing, we identified a heterozygous missense mutation in the gene *CSPG4* which is responsible for coding the protein NG2. This protein is involved in the maturation of the oligodendrocyte precursor cells (OPCs) which are involved in the development and maturation of the oligodendrocytes. Oligodendrocytes ensheath axons to ensure fast transmission of neuronal information and maximize the efficiency of energy utilization. We identified multiple other families with other rare coding variants in *CSPG4* and provide converging evidence for pathogenicity based on brain imaging of the discovery family members and induced pluripotent stem cell (IPSC)-derived oligodendrocyte precursor cell (OPC) experiments.

In chapter **three**, we describe a similar approach in a large Dutch family with bipolar disorder. Through linkage analysis coupled with exome sequencing, we identified a rare missense variant in the gene *GRM2* coding for the metabotropic glutamate receptor 2 protein (mGluR2). mGluR2 is thought to be involved in the pathogenesis of psychosis through reduced

modulation of the excitotoxicity of glutamate. mGluR2 is expressed on the presynaptic terminals and postsynaptic spines. Through functional assays, we were able to determine the variant has a loss of function effect on the protein. mGluR2 has been a protein of interest for the pharmaceutical industry in the past years for the development of antipsychotic compounds modulating the signalling of the metabotropic glutamate receptor, type 2.

In the second section, we describe two families with neurodevelopmental disorders. In chapter **four**, we describe a Dutch consanguineous family with five sons, four them with ASD. We identified a compound heterozygous variant in the gene *SLC39A7*. Existing literature suggests the ZIP7 protein encoded by this gene is very important in brain development and its malfunction causes neurodevelopmental abnormalities. In chapter **five**, we describe a consanguineous Arab Israeli family of Bedouin descent. Through linkage analysis coupled with exome sequencing, we identified a homozygous mutation in the gene *ACO2,* which codes for mitochondrial aconitase. This is an essential enzyme in the tricarboxylic acid (TCA) cycle, which is the main source of energy for living organisms.

In the third section, we cover syndromic psychiatric phenotypes and chromosomal aberrations and their role in psychiatric disorders. In chapter **six** we describe a family with multiple members with bipolar disorder and miscarriages. Through karyotyping and whole-genome sequencing with Sanger confirmation, we identified a segregating balanced translocation with two intragenic breakpoints disrupting *BCL2L10* and *PNLDC1*. We propose both genes as candidates for affective psychosis (bipolar disorder and schizoaffective disorder).
In the fourth section, we discuss what these findings mean for our field.
In chapter **seven** we describe the outcomes of two cohorts of consecutive psychiatric inpatients with a syndromic phenotype who were seen for clinical genetic consultation and genetic testing. In a significant percentage of the patients, we were able to identify rare chromosomal aberrations causally or potentially causally linked to the patients' phenotypes. We call for implementation of clinical genetic consultation and testing in adult psychiatric patients with a syndromic presentation.
Chapter **eight** is an essay describing the psychodynamics of families heavily loaded for severe psychiatric disorders. In chapter **nine**, we provide a general discussion on the topic of genetics in the field of psychiatry.

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Part I

Adult-onset psychiatric disorders

2 |

CSPG4 mutations and induced pluripotent cell modeling implicate oligodendrocyte progenitor cell dysfunction in familial schizophrenia

Femke M. de Vrij, PhD[1†], Christian G. Bouwkamp, MSc[1,2†], Nilhan Gunhanlar, PhD[1†], Guy Shpak, PhD[1], Bas Lendemeijer, MSc[1], Maarouf Baghdadi, MSc[1], Mehrnaz Ghazvini, PhD[3], Tracy M. Li, BSc, Marialuisa Quadri, PhD[2], Simone Olgiati, PhD[2], Guido J. Breedveld, BSc[2], Michiel Coesmans, MD, PhD[1,12], Edwin Mientjes, PhD[9,10], Ton de Wit, BSc[2], Frans W. Verheijen, PhD[2], H. Berna Beverloo, PhD[2], Dan Cohen, MD, PhD[13], Rob M. Kok, MD, PhD[14], P.Roberto Bakker, MD, PhD[15,16], Aviva Nijburg, MD[14], Annet T. Spijker, MD, PhD[14], P.M.Judith Haffmans, PhD[17,18], Erik Hoencamp, MD, PhD[14,18], Veerle Bergink, MD, PhD[1], GROUP Study Consortium[#], Jacob A. Vorstman, MD, PhD[19], Timothy Wu, BSc[20], Loes M. Olde Loohuis, PhD[20], Najaf Amin, PhD[4], Carolyn D. Langen, MSc[6,7,8], Albert Hofman, MD, PhD[4], Witte J. Hoogendijk, MD, PhD[1], Cornelia M. van Duijn, PhD[4], M. Arfan Ikram, MD, PhD[4,5,6], Meike W. Vernooij, MD, PhD[4,6], Henning Tiemeier, MD, PhD[1,4,7], André G. Uitterlinden, PhD[4,11], Ype Elgersma, PhD[9,10], Ben Distel, PhD[21,9], Joost Gribnau, PhD[3], Tonya White, MD, PhD[7], Vincenzo Bonifati, MD, PhD[2], Steven A. Kushner, MD, PhD[1*]

[†] *These authors contributed equally*

[#] *The investigators in the GROUP study consortium are listed in the Supplement.*

Manuscript submitted

Affiliations:

[1] Department of Psychiatry, Erasmus Medical Center, Rotterdam, The Netherlands

[2] Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, The Netherlands

[3] Department of Developmental Biology, and Erasmus MC iPS Facility, Erasmus Medical Center, Rotterdam, The Netherlands

[4] Department of Epidemiology, Erasmus Medical Center, Rotterdam, The Netherlands

[5] Department of Neurology, Erasmus Medical Center, Rotterdam, The Netherlands

[6] Department of Radiology, Erasmus Medical Center, Rotterdam, The Netherlands

[7] Department of Medical Informatics, Erasmus Medical Center, Rotterdam, The Netherlands

[8] Biomedical Imaging Group Rotterdam, Departments of Radiology & Medical Informatics, Erasmus Medical Center, Rotterdam, The Netherlands

[9] Department of Neuroscience, Erasmus Medical Center, Rotterdam, The Netherlands

[10] ENCORE Expertise Centre For Neurodevelopmental Disorders, Erasmus Medical Center, Rotterdam, The Netherlands

[11] Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands

[12] Delta Psychiatric Center, Poortugaal, The Netherlands

[13] Mental Health Care Organization North-Holland North, Heerhugowaard, The Netherlands

[14] Parnassia Psychiatric Centre, The Hague, The Netherlands

[15] Department of Psychiatry and Psychology, School of Mental Health and Neuroscience, Maastricht University Medical Center, Maastricht, The Netherlands

[16] Psychiatric Center GGZ Centraal, Amersfoort, The Netherlands

[17] PsyQ, The Hague, The Netherlands

[18] Institute of Psychology, Leiden University, The Netherlands

[19] Department of Psychiatry, Brain Center Rudolf Magnus, University Medical Center Utrecht, The Netherlands

[20] Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, University of California, Los Angeles, CA, USA

[21] Department of Medical Biochemistry, Academic Medical Centre, Amsterdam, The Netherlands

ABSTRACT

Importance: Schizophrenia is highly heritable, yet its underlying pathophysiology remains largely unknown. Among the most well replicated findings in neurobiological studies of schizophrenia are deficits in myelination and white matter integrity, however direct etiological genetic and cellular evidence has thus far been lacking.

Objective: To implement a family-based approach for genetic discovery in schizophrenia combined with functional analysis using induced pluripotent stem cells (iPSCs).

Design, Setting, and Participants: Exome sequencing was performed in a large multiplex discovery pedigree, followed by targeted resequencing of probands from 73 independent multiplex families with schizophrenia. Case-control validation was implemented using the Swedish Schizophrenia Exome Sequencing Study case-control cohort (2512 cases, 2578 controls). iPSCs were derived from 3 affected and 3 unaffected siblings of the discovery family.

Main Outcomes and Measures: Familial segregation with schizophrenia of rare exome variants and case-control validation. Functional cellular analyses of lineage-specific iPSC differentiation were performed using whole-cell electrophysiological recordings, protein biochemistry, and cellular morphology.

Results: We observed two rare missense mutations in *Chondroitin Sulfate Proteoglycan 4* (*CSPG4*) associated with familial schizophrenia (c.391G>A [p.A131T], MAF 7.79 x 10^{-5} and c.2702T>G [p.V901G], MAF 2.51 x 10^{-3}). The $CSPG4^{A131T}$ mutation was absent from the independent case-control cohort, and nominally enriched in cases for the $CSPG4^{V901G}$ mutation (11 cases vs. 3 controls, P = 0.026, OR 3.77, 95% CI 1.05-13.52). CSPG4 is a hallmark protein of oligodendrocyte progenitor cells (OPCs). IPSC-derived neurons exhibited similar intrinsic excitability and synaptic activity in $CSPG4^{A131T}$ mutation carriers and their non-carrier siblings. In contrast, iPSC-derived OPCs from mutation carriers exhibited abnormal post-translational processing (P = 0.029) and subcellular localization of mutant NG2 (P = 0.007), as well as aberrant cellular morphology (P = 3.0 x 10^{-8}). Moreover, *in vivo* diffusion tensor imaging of both available $CSPG4^{A131T}$ mutation carriers demonstrated a significant reduction of brain white matter integrity compared to unaffected sibling and matched general population controls (P = 2.2 x 10^{-5}).

Conclusion and Relevance: Our findings provide a convergence of genetic and functional evidence to implicate OPC dysfunction as a candidate pathophysiological mechanism of familial schizophrenia.

INTRODUCTION

Schizophrenia is a severely debilitating psychiatric disorder affecting ~1% of the population worldwide[1]. The strongest known determinant for developing schizophrenia is family history. A meta-analysis which included five decades of twin studies concluded a heritability estimate (h^2) of 0.77 ± 0.05, with a relatively limited contribution of shared environmental influences (c^2) (0.013 ± 0.025)[2].

The Psychiatric Genomics Consortium recently reported a genome-wide association study (GWAS) investigating 36,989 cases and 113,075 controls, in which 128 genome-wide significant single-nucleotide variants were identified across 108 independent genomic loci, suggesting an important contribution of common genetic variation to schizophrenia risk[3]. However, a large proportion of the heritability for schizophrenia remains unexplained, leaving many genetic variants remaining to be discovered. Therefore, increasing attention has also been focused on the potential contribution of rare genomic variation to schizophrenia risk. Copy number variants (CNVs) are a well established source of pleiotropic risk, ranging from asymptomatic carriership to a complex constellation of symptoms affecting multiple organ systems, such as in the case of the 22q11.2 microdeletion syndrome[4]. In a large-scale schizophrenia case-control cohort analysis, known pathogenic CNVs were significantly more frequent in cases (2%) than controls (0.4%)[5,6]. Moreover, an independent study found that large (>500kb) CNVs are enriched in loci associated with schizophrenia by GWAS and frequently involve genes encoding proteins located in the postsynaptic density[7].

In addition to microarray-based genotyping methods, the development of next-generation sequencing has allowed the possibility to examine whether rare single nucleotide variants or small insertions-deletions contribute to schizophrenia risk. In the largest case-control study to date using whole-exome sequencing, a Swedish cohort including 2,536 cases and 2,543 controls yielded no single mutation or single gene reaching genome-wide significance for association with schizophrenia, but confirmed a similar enrichment of gene sets for synaptic function as previously identified for genes located in schizophrenia-associated CNVs and GWAS loci[8]. Moreover, trio-based studies have identified a number of candidate genes through identification of recurrent *de novo* mutations[9,10] and an increased burden of mutations occurring in genes encoding glutamatergic postsynaptic proteins[11].

Recent genetic and induced pluripotent stem cell (iPSC)-based studies have converged on a model by which neuronal function, and in particular synaptic transmission, is a major pathophysiological mechanism of schizophrenia[3,8,11–13]. However, functional neuronal

alterations may arise either by direct cell-type autonomous changes to neurons themselves, or indirectly through a primary pathophysiological influence on other cell types that influence neuronal function. Numerous studies have reported the involvement of glial cell biology in the pathophysiology of schizophrenia, including alterations in oligodendrocytes, myelination, and white matter integrity[14–19], which directly regulate neuronal function.

Abnormalities of the integrity of the white matter are strongly associated with schizophrenia[20]. The late adolescent critical period for cerebral cortex myelination has long been recognized as overlapping closely with the typical age of onset for schizophrenia[16,17]. Myelination-related genes have been shown to be enriched for common variants associated independently to white matter integrity[21] and schizophrenia[15,22]. Two recent brain imaging studies have elegantly compared white matter integrity in 16p11.2 deletion and duplication carriers[23,24] of which only the 16p11.2 duplication confers increased risk for schizophrenia[6]. Notably, both global fractional anisotropy and white matter volume were selectively decreased in 16p11.2 duplication carriers. However, despite increasing evidence of an association between schizophrenia and myelination integrity, the molecular and cellular mechanisms by which oligodendrocyte lineage dysfunction might influence schizophrenia risk have remained largely unknown.

We now report genetic and functional evidence of oligodendrocyte progenitor cell dysfunction in schizophrenia. Using a family-based genetic approach, we observed multiple rare missense mutations in *CSPG4* that segregate with schizophrenia. Using iPSCs reprogrammed from affected *CSPG4* mutation carriers and their unaffected non-carrier siblings, we demonstrate that patient-derived OPCs exhibit abnormal post-translational processing, aberrant subcellular localization of CSPG4/NG2, and abnormal cellular morphology. Moreover, diffusion tensor imaging of *CSPG4* mutation carriers exhibited a global impairment in white matter integrity, together providing support for OPC dysfunction as a candidate pathophysiological mechanism of schizophrenia.

RESULTS

Genetic findings in the discovery family

A non-consanguineous family of Dutch ancestry was ascertained with a pattern of schizophrenia inheritance compatible with autosomal dominant transmission. The core pedigree consisted of a couple and their nine children (5 males, 4 females) of whom the father

and 4 sons suffered from non-syndromic schizophrenia (**Figure. 1A** and see **eTable 1** in Supplement).

Genome-wide parametric linkage analysis was performed on peripheral blood DNA using an autosomal-dominant, affected-only model of inheritance, in order to identify genomic regions shared among all affected family members (see **eTable 2** in Supplement). Whole exome sequencing was performed on three individuals of the family (pedigree IDs: II-2, III-5 and III-9; **Figure 1A**). Five candidate heterozygous variants were identified based on the following criteria: a) located within the genomic regions shared among all affected family members, b) predicted to affect protein coding (missense, nonsense, frameshift, splice site), c) called in at least one of the affected individuals [III-5 and III-9], d) absent from the unaffected mother [II-2], e) absent from dbSNP129, and f) with a minor allele frequency (MAF) < 0.001 in the Exome Aggregation Consortium (ExAC) browser (Europeans non-Finnish)[25], EVS6500 European Americans, NHLBI Exome Sequencing Project (ESP)[26], 1000 Genomes[27], and Genome of the Netherlands (GoNL)[28] cohorts (see **eTable 3** in Supplement). Genotyping of these variants was performed by Sanger sequencing in all participating family members.

Among the five candidate variants, *CSPG4* c.391G>A (p.A131T) was the only variant shared by all affected family members and absent in all unaffected relatives, including in the extended family (**Figure 1A** and **B**). *CSPG4* c.391G>A (p.A131T) is present in the Exome Aggregation Consortium Browser (Total [forward strand]: T=6/C=118,148 alleles [MAF 5.08 x 10^{-5}], European (Non-Finnish): T=5/C=64,215 alleles [MAF 7.79 x 10^{-5}])[25] but absent from the Swedish Schizophrenia Population-Based Case-control Exome Sequencing Study (2536 cases, 2543 controls)[8], 1000 Genomes[27], and Genome of the Netherlands (GoNL)[28].

Additional genotyping of CSPG4 discovery family variant

In an effort to further characterize the frequency of *CSPG4* c.391G>A (p.A131T) in the Netherlands, we performed TaqMan genotyping and Sanger sequencing validation in an independent Dutch cohort of 1219 schizophrenia cases and in the general population-based Rotterdam Study cohort[29] (10,611 subjects). One carrier was identified among the schizophrenia cases (MAF_{cases} 4.1 x 10^{-4}) and three within the general population ($MAF_{population}$ 1.4 x 10^{-4}). The patient carrier had a long history of severe psychiatric illness including multiple hospital admissions and chronic anti-psychotic and anti-depressant medication. Her most recent prescriptions included penfluridol oral depot (20 mg/week) and venlafaxine (37.5 mg/day).

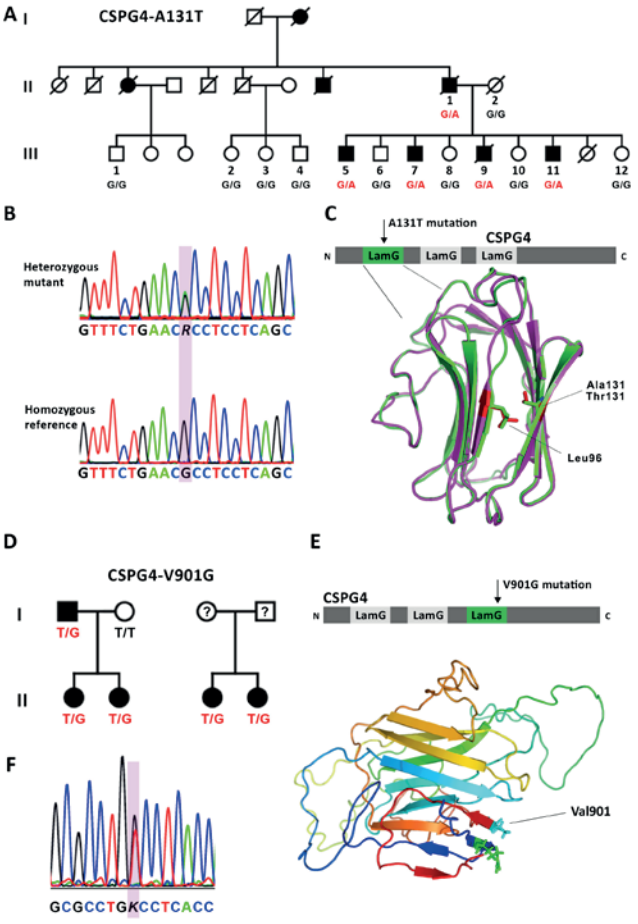

Figure 1. Familial segregation of *CSPG4* mutations with schizophrenia. A: Pedigree of multiplex discovery family with schizophrenia. Symbols: filled, schizophrenia; open, unaffected; G/A, heterozygous carrier of the *CSPG4* c.391G>A mutation (*CSPG4*^{A131T}); G/G, homozygous reference. **B:** Representative sequencing results for heterozygous carriers of the *CSPG4* c.391G>A mutation. The lower panel reflects homozygous reference sequence. **C:** Homology model of the first Laminin G domain of CSPG4. Structural alignment of the reference (green) and mutant model (magenta) reveals a difference in the predicted interaction between amino acid positions 131 and 96 (Leu) in the opposing β-strand inside the hydrophobic core of the β-sandwich (predicted alterations of the side-chains in red). **D:** Family pedigrees segregating the *CSPG4* c.2702T>G mutation (*CSPG4*^{V901G}). Symbols: filled, schizophrenia; open, unaffected; T/G, heterozygous carrier of the *CSPG4* c.2702T>G mutation; T/T, homozygous reference. **E:** Three-dimensional structural homology modeling of the putative 3rd LamG domain of CSPG4 (a.a. 634-921), demonstrating the outside surface location of Val^{901}. **F:** Representative Sanger sequencing trace of heterozygous carrier of the *CSPG4* c.2702T>G mutation.

Family members could not be ascertained for additional psychiatric history or genotyping. Among the three unrelated carriers (maximum pairwise $\hat{\pi}$ = 0.078) identified in the general population cohort, two had a clinically significant history of psychiatric illness ($\hat{\pi}$ = 0.055). One of the subjects had a history of multiple inpatient psychiatric hospitalizations for depression, and the other required chronic antidepressant and anxiolytic pharmacotherapy. Notably these findings were unlikely due to chance alone, given the 12.76% period prevalence of antidepressant use in the Rotterdam Study cohort and 0.065% annual prevalence of inpatient psychiatric hospitalization in the Netherlands[30,31] (Binomial $P = 8.8 \times 10^{-3}$).

Identification of second rare CSPG4 variant that segregates with schizophrenia

A previous study identified suggestive linkage at chromosome 15q22-24, containing *CSPG4,* in a cohort of 175 families with schizophrenia or schizoaffective disorder of Central American/Hispanic origin[32,33]. We therefore sequenced the full open reading frame of *CSPG4* in one proband from each of the 73 families that positively contributed to the linkage signal at this locus (markers D15S131 and D15S655) (see **eTable 4** in Supplement). Four rare missense variants were identified with MAF < 0.005 (ExAC Browser Latino) (**Figure 1**, and see **eFigure 1** in Supplement). However, *CSPG4* c.2702T>G (p.V901G) was the only variant found in two independent families, without evidence of incomplete penetrance (**Figure 1D, F**), and located within a LamG domain similar to the c.391G>A (p.A131T) discovery family variant (**Figure 1E**). The association of the c.2702T>G p.V901G variant with schizophrenia was further supported by the Swedish Schizophrenia Population-Based case-control Exome Sequencing Study[8] (MAF_{cases} 2.19 x 10^{-3}; $MAF_{controls}$ 5.82 x 10^{-4}, Fisher's Exact Test P = 0.026, OR 3.77, 95% CI 1.05-13.52). Together, these findings suggest that rare coding variants of *CSPG4* contribute to schizophrenia liability.

Structural modeling of CSPG4 mutations

The $CSPG4^{A131T}$ and $CSPG4^{V901G}$ mutations are located within the first and third Laminin G domain of the protein encoded by *CSPG4*, known as neural/glial 2 (NG2) (**Figure 1C, E**). Laminin G (LamG) domains are highly conserved and present in a diverse group of extracellular matrix proteins[34]. Intriguingly, several schizophrenia-associated genes such as *NRXN1* and *LAMA2* also contain LamG domains[10,35–37]. The presence of several crystal structures of LamG domains in the Protein Data Bank allowed homology modeling of the LamG domains of NG2. Models implemented using Phyre2[38] and I-TASSER [39] both suggested

that in the reference sequence, Ala[131] and Leu[96] interact across opposing β-sheets inside the hydrophobic core of the β-sandwich. The mutation of Ala[131], which has a small hydrophobic side chain, to Thr[131], containing a larger polar side-chain, suggested a conformational change impairing the proper folding of the β-sandwich (**Figure 1C**).

Interestingly, *CSPG4*^{V901G} is located in a putative 3rd LamG domain predicted by I-TASSER [39] (a.a. 634-921, **Figure 1E**). This region has not previously been annotated as a LamG domain, despite the striking structural homology to other LamG domains with available crystal structures, most notably that of NRXN1[40–42]. In contrast to the *CSPG4*^{A131T} mutation that is located on the inside of the globular structure of the first LamG domain of NG2, the *CSPG4*^{V901G} mutation is predicted to be located on the outside of the putative third LamG domain (**Figure 1E**), therefore perhaps affecting protein-protein interactions. Intriguingly, the same protein region has been found to bind to collagen V and VI, implicated in cell adhesion and migration[43,44].

Family-based iPSC modeling of the CSPG4^{A131T} *mutation*

No evidence for a cell-autonomous neuronal phenotype

Recent genetic and iPSC-based studies of schizophrenia have converged on a model by which neuronal function, and in particular synaptic transmission, is a major pathophysiological mechanism[3,8,11–13]. We obtained skin biopsies for iPSC reprogramming from three affected *CSPG4*^{A131T} carriers and three unaffected non-carriers within the core sibship of the discovery family (see **eFigure 2 A-D** in Supplement). Directed differentiation of iPSCs yielded forebrain-specified neural progenitor cells (NPCs) uniformly positive for Nestin, SOX2, Vimentin, and FOXG1 (see **eFigure 2E** in Supplement).

NPCs were differentiated to neural cultures for 8-10 weeks, which notably lack cells of the oligodendrocyte lineage including OPCs. Both control and patient-derived neurons developed robust synaptic network connectivity, confirmed by confocal immunofluorescence (**Figure 2, A-C**) and whole-cell patch-clamp electrophysiological recordings (**Figure 2, D-R**). Overall, neurons derived from patient carriers and their unaffected siblings had similar electrophysiological properties, including passive membrane properties, action potential characteristics, and synaptic physiology. Of the twelve different electrophysiological parameters examined, input resistance (t_{50}=2.54, P=0.01) and action potential threshold (t_{49}=2.84, P=0.007) exhibited nominally significant differences that did not survive Bonferroni

correction. Accordingly, the lack of a robust neuronal phenotype is consistent with the absence of expression of NG2 in neurons[45].

Abnormal posttranslational processing and subcellular localization of CSPG4^{A131T} in OPCs

Given the highly abundant expression of NG2 in OPCs, widely referred to as NG2 cells, we next sought to investigate the influence of the *CSPG4^{A131T}* mutation on iPSC-derived OPCs. Directed differentiation of iPSCs to OPCs resulted in robust expression of the lineage specific markers NG2, PDGFRa, Olig2, and SOX10 (see **eFigure 3** in Supplement). We first examined the subcellular distribution of NG2, as the structural homology modeling of the *CSPG4^{A131T}* mutation suggested aberrant protein folding (**Figure 1C**). Since NG2 is a transmembrane protein, it requires processing by the secretory pathway. Consistent with an impairment of protein processing, *CSPG4^{A131T}* patient-derived OPCs showed a highly abnormal subcellular localization of NG2 exemplified by an increase of co-localization with the endoplasmic reticulum marker calreticulin (t=5.08, P = 0.007) (**Figure 3, A and B**).

In order to further characterize the alteration of NG2 subcellular localization, we performed surface biotinylation of *CSPG4^{A131T}* patient and non-carrier sibling control OPCs. NG2 is known to undergo extensive posttranslational modification through the addition of chondroitin sulphate moieties throughout the protein[46,47]. Consequently, NG2 appears as multiple bands by western blotting: a sharp band at 300 kDa corresponding to an unmodified form of NG2 which lacks chondroitin sulfate side chains, and a large polydisperse smear at >300 kDa corresponding to NG2 with chondroitin sulfate modification. Pre-incubation with chondroitinase ABC to enzymatically cleave the chondroitin sulfate side chains eliminated the >300 kDa polydisperse smear (modified NG2) and increased the 300 kDa band (unmodified NG2) (**Figure 3C**).

The total level of NG2 protein was similar between patient and control OPCs in whole cell lysates (t=0.51, P = 0.62) (**Figure 3D,** see **eFigure 4** and **eFigure 5** in Supplement). However, patient OPCs exhibited a significant decrease in the ratio of modified versus unmodified NG2 compared to control OPCs. This finding was observed in whole cell lysate (t=2.88, P=0.04), as well as independently in the intracellular (t=3.50, P=0.02) and surface protein fractions of OPCs (t=3.31, P=0.03) (**Figure 3, D and E**). Taken together, these results demonstrate that the *CSPG4^{A131T}* mutation results in abnormal processing of NG2 protein.

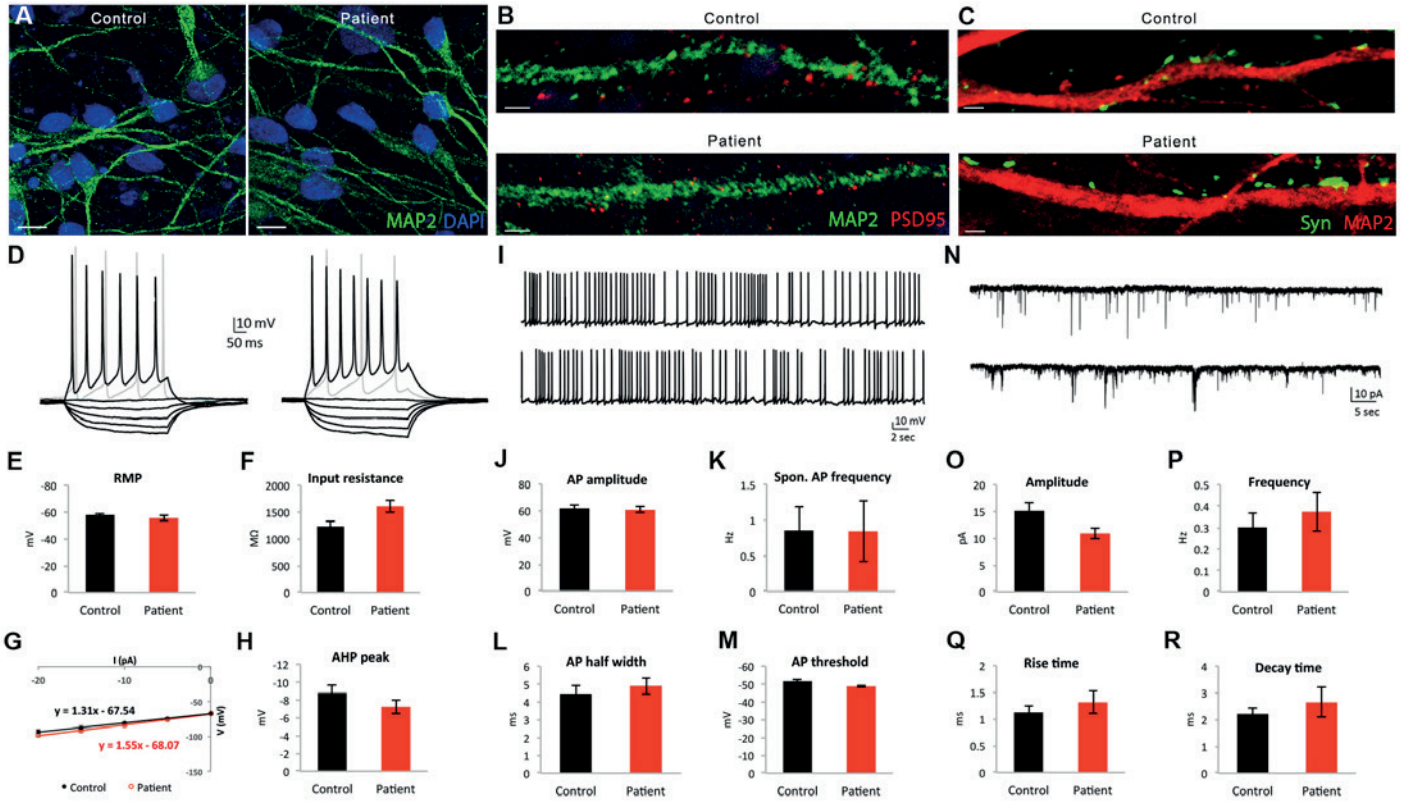

Figure 2. Normal passive, active, and synaptic function in *CSPG4*^{A131T} patient iPSC-derived neurons. A: Immunostaining of iPSC-derived neural cultures after 8 weeks of differentiation (scale bar = 10 μm). **B, C**: Immunostaining with MAP2, PSD95 and synapsin antibodies confirmed the presence of synaptic proteins on dendrites of iPSC-derived neurons 8 weeks post-differentiation (scale bar = 2 μm). **D-R**: Electrophysiological measurements of iPSC-derived neurons. **D**: Representative voltage responses to hyperpolarizing (-20 – 0 pA, 5 pA increments) and depolarizing (10 and 20 pA) current steps (left: control, right: patient). **E**: Resting membrane potential (RMP) (t_{44}=1.04, P=0.30). **F:** Input resistance

(t_{50}=2.54, P=0.01). **G**: Current-voltage (I-V) relationship of patient and control cells. **H**: AHP peak (t_{49}=1.35, P=0.18). **I**: Representative traces of spontaneous action potential (AP) firing (50 sec at RMP; top: control, bottom: patient). **J**: AP amplitude (t_{49}=0.31, P=0.76). **K**: Spontaneous firing rate (t_{19}=0.03, P=0.98). **L**: AP half width (t_{49}=0.65, P=0.52). **M**: AP voltage threshold (t_{49}=2.84, P=0.007). **N**: Representative traces of spontaneous postsynaptic currents (100 sec at -90 mV; top: control, bottom: patient). **O**: sPSC amplitude (t_{17}=1.94, P=0.07). **P**: sPSC frequency (t_{17}=0.65, P=0.52). **Q**: sPSC rise time (t_{17}=0.84, P=0.41). **R**: sPSC decay time (t_{17}=0.84, P=0.41). **D-R**, Unpaired two-tailed Student's t-test. Passive properties and evoked APs (N=24 control, N=28 patient). Spontaneous APs (N=11 control, N=9 patient). Spontaneous postsynaptic currents (N=12 control, N=7 patient). All error bars are +/- standard errors of the mean (SEM).

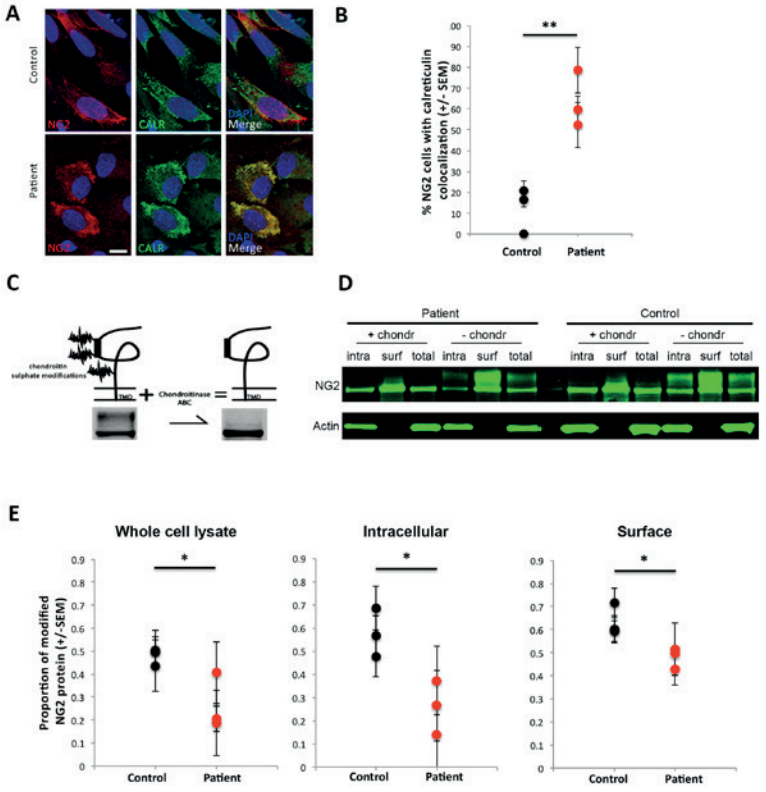

Figure 3. Aberrant subcellular localization and post-translational chondroitin sulphate modification of NG2 in *CSPG4*^{A131T} patient-derived OPCs. A, B: Immunostaining for NG2 and calreticulin (CALR) reveals increased ER colocalization of NG2 in patient OPCs (scale bar = 10 μm). Quantification was performed in two independent experiments with three control and three patient OPC lines each. **C:** Chondroitin sulphate side chains are specifically cleaved by chondroitinase treatment. **D:** Representative western blot of biotinylation assay samples of control and patient OPCs +/- chondroitinase ABC treatment (intra = intracellular fraction, surf = surface fraction, total = whole cell lysate fraction). **E:** Quantification of the proportion of chondroitin sulphate-modified NG2 protein. The proportion of modified to unmodified NG2 in OPC extracts was determined by normalizing the integrated density of modified NG2 (>300 kDa) to the total integrated density of the modified plus unmodified NG2 bands. Experiments using control (n=9) and patient (n=8) OPCs are shown as aggregated data per individual subject (3 control and 3 patient siblings). Error bars in all panels reflect standard errors of the mean. Unpaired two-tailed Student's t-test revealed significantly less modified NG2 protein in whole cell lysate (t=2.88, P=0.04), intracellular (t=3.50, P=0.02) and surface fractions (t=3.31, P=0.03).

Abnormal morphology of OPCs derived from CSPG4^{A131T} *mutation carriers*

In addition to the abnormal processing of NG2 protein, we also observed distinct morphological differences between OPCs derived from patients and controls (**Figure 4A**).

Patient-derived OPCs exhibited a size distribution that was strongly shifted towards smaller cells, a finding that was highly significant across all patient and control lines (Kolmogorov-Smirnov D=0.25, P=3.0 x 10^{-8}; **Figure 4B**). Overall, these results suggest that abnormal processing of NG2 influences the function of OPCs derived from *CSPG4*^{A131T} mutation carriers.

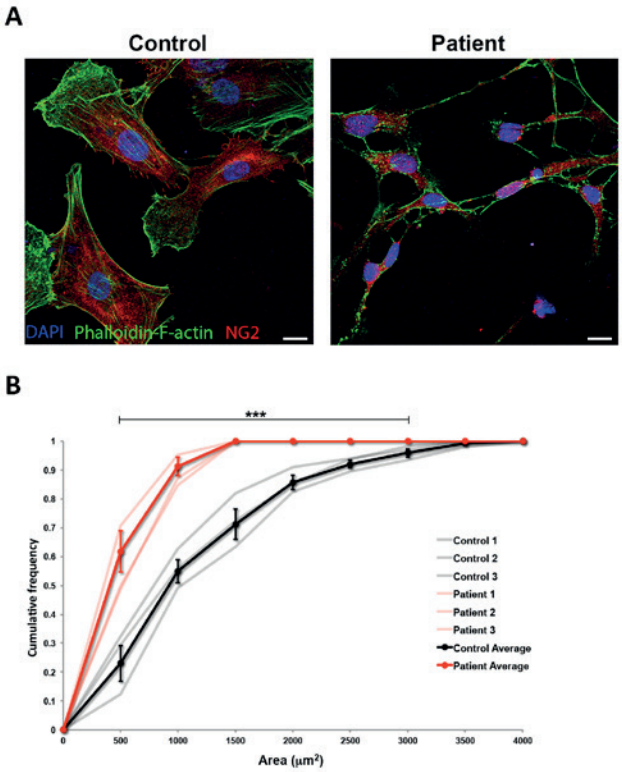

Figure 4. Abnormal morphology of OPCs derived from *CSPG4*^{A131T} mutation carriers. A: OPCs derived from mutation carriers exhibit an abnormally small morphology (scale bars = 10 μm). **B:** Cumulative distribution of OPC area (μm²; n=361 control cells, n=217 patient cells) demonstrates the significant reduction in size of OPCs derived from affected mutation carriers compared to their unaffected siblings. Control and patient cell size distributions were compared by Kolmogorov-Smirnov test (D=0.25, P=3.0 x 10^{-8}). Dark lines show group mean +/- standard error. Grey and pink lines show the results from each of the 3 individual control and patient subjects, respectively.

Impaired white matter microstructure in *CSPG4*^{A131T} mutation carriers

Given the observed abnormalities of patient-derived OPCs, we reasoned that affected *CSPG4*^{A131T} mutation carriers might exhibit impairments of white matter integrity since OPCs are the exclusive precursor of myelinating oligodendrocytes. Therefore, we performed brain

magnetic resonance imaging (MRI)-based diffusion tensor imaging (DTI) in affected carrier and unaffected non-carrier siblings and compared them with 294 subjects from the general population Rotterdam Study cohort matched for age, gender, smoking behavior, and alcohol use (**Figure 5A**). DTI images were analysed for global and focal reductions in fractional anisotropy (FA), the latter referred to as white matter potholes[48]. Consistent with the hypothesis that the *CSPG4*^{A131T} mutation compromises the integrity of myelination, affected carriers exhibited both a significantly higher number of white matter potholes (P = 2.2 x 10^{-5}) and lower global FA (P = 8.2 x 10^{-3}), compared to unaffected sibling and matched general population controls (**Figure 5, B and C**).

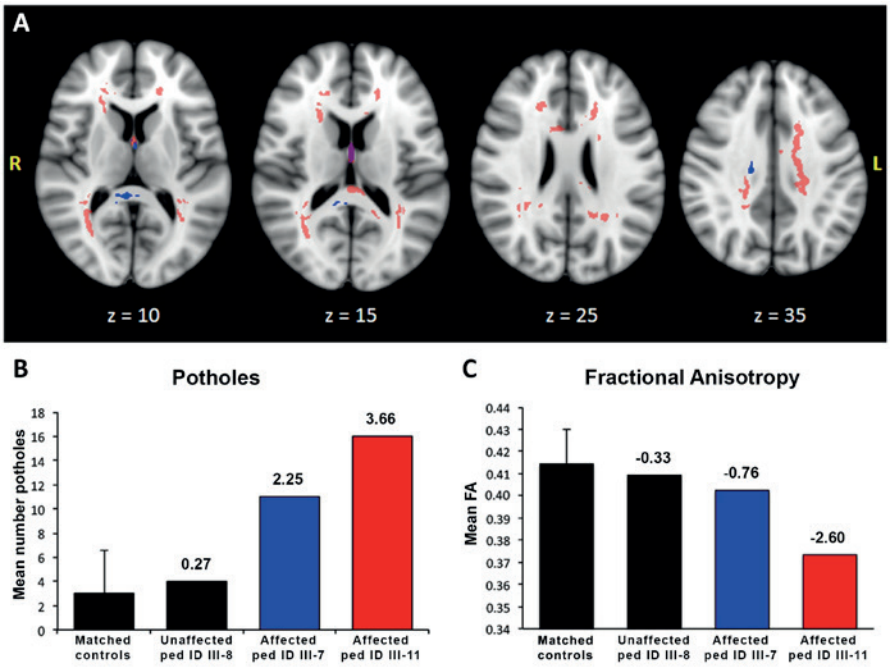

Figure 5. White matter abnormalities in *CSPG4*^{A131T} patients. A: White matter potholes found in the two affected family members are shown in red and blue, respectively. Purple regions define a spatial overlap of potholes in both patients. The z-measures provide coordinates of the axial plane in MNI-space. **B:** Mean number of potholes comparing the matched control population to the unaffected and two affected family members. **C:** Whole-brain white matter fractional anisotropy (FA) comparing the matched general population subjects to unaffected and affected family members. Error bars in **B** and **C** reflect the standard deviation of the matched general population subjects. The number above each bar reflects the individual z-score compared to the matched general population group.

DISCUSSION

Our findings provide evidence that oligodendrocyte progenitor cell dysfunction might influence the pathophysiology of schizophrenia. We identified two different rare missense mutations in *CSPG4* exhibiting familial segregation with schizophrenia. The discovery family *CSPG4*^{A131T} mutation was associated with impaired protein processing and abnormal OPC morphology in oligodendrocyte precursor cells. Moreover, *CSPG4*^{A131T} mutation carriers were found to have impaired white matter microstructure suggestive of compromised myelin integrity. A second mutation (*CSPG4*^{V901G}) also located in a LamG domain was observed in two independent pedigrees with nominally significant case/control enrichment in the Sweden schizophrenia exome study[8].

Our genetic findings highlight some of the challenges frequently encountered in the effort to identify pathogenic rare variants underlying common diseases such as schizophrenia. Although we identified three independent families exhibiting segregation of two different rare *CSPG4* missense variants with complete penetrance, none of these families was large enough on its own to achieve a genome-wide significant LOD score. Moreover, although the rare MAF of the *CSPG4*^{A131T} mutation precluded case/control validation in the Sweden schizophrenia exome study cohort since it was absent from both cases and controls totaling more than 5000 people, two of the three *CSPG4*^{A131T} mutation carriers in the general population Rotterdam Study cohort had a clinically significant history of psychiatric illness that was unlikely due to chance. Furthermore, the *CSPG4*^{V901G} mutation was present in the Swedish cohort with a nominally significant enrichment in cases versus controls. Additional follow-up studies in larger cohorts will be required to definitively evaluate the association between schizophrenia and rare *CSPG4* variants.

Genetic variation in *CSPG4* might have pleiotropic effects since two of the *CSPG4*^{A131T} mutation carriers identified in the general population Rotterdam Study cohort had a clinically significant history of depression. Interestingly, a mouse model of OPC depletion was recently shown to exhibit depression-like behavior[49]. Such pleiotropic influences on mental health outcomes would be consistent with many of the previously identified genetic risk factors for schizophrenia[50]. Furthermore, originally named as melanoma-associated chondroitin sulfate proteoglycan upon its cloning[51], NG2/CSPG4 has since been widely implicated in a wide variety of human cancers both as a diagnostic marker and a therapeutic target, including for glioblastoma and melanoma[52,53].

The protein sequence surrounding the *CSPG4*^{A131T} mutation is conserved only among higher-order primates, suggesting recent evolutionary pressure. In contrast, the *CSPG4*^{V901G} variant is predicted to be a disease-causing mutation by PolyPhen2[54]. In non-primate vertebrates, the reference amino acid at position 131 is threonine, corresponding to the patient mutation *CSPG4*^{A131T}, contributing to a benign PolyPhen score. Interestingly, this observation is analogous to the human *SNCA*^{A53T} mutation, one of the most well established mutations for autosomal dominant Parkinson's Disease[55]. In rodents, the reference amino acid at *SCNA* position 53 is a threonine. However, although transgenic expression of the reference human *SCNA* sequence in mice is benign, introduction of the human *SNCA*^{A53T} mutation is highly pathogenic[55].

To investigate the cellular pathophysiology resulting from mutation of *CSPG4,* we derived iPSCs from affected and unaffected siblings of the discovery family. Detailed electrophysiological analysis of patient-derived neurons did not reveal a robust phenotype, consistent with the lack of expression of *CSPG4* in neurons and the absence of OPCs from the neuronal cultures. In contrast, multiple previous studies have demonstrated direct functional neuronal mechanisms in human iPSC models of schizophrenia[3,8,11–13,56]. Importantly, our findings do not imply that neuronal dysfunction is not central to the pathophysiology of schizophrenia. Rather, these data suggest that although the symptoms of schizophrenia are ultimately manifest from neuronal dysfunction, the primary pathophysiological mechanism could be mediated by either direct neuronal impairments or indirectly through non-neuronal cell types including OPCs.

Although the precise mechanism remains to be elucidated, alterations in myelination are a parsimonious candidate given our convergent findings of genetic mutations in the OPC marker protein *CSPG4*, functional impairments of iPSC-derived OPCs, and *in vivo* DTI-based structural brain imaging. Taken together, these findings are highly consistent with the growing body of evidence implicating white matter integrity in schizophrenia neuropathology[48,57–59]. Moreover, our results demonstrate abnormal subcellular localization of NG2 in patient-derived OPCs. Notably, cells of the oligodendrocyte lineage are known to be particularly susceptible to disruptions in the secretory pathway, as their maturation requires a substantial upregulation of membrane protein expression[60].

OPCs receive extensive GABAergic input from surrounding neurons, which regulate their differentiation to myelinating oligodendrocytes[61–64]. However, it has remained less well understood whether OPCs directly modulate neuronal function independent of myelination.

Notably, two recent studies have suggested novel candidate mechanisms by which OPC dysfunction might directly regulate neuronal function, including activity-dependent ectodomain cleavage of NG2[47] and local buffering of extracellular potassium[65].

In summary, our findings support the validity of family-based genetics and iPSC modeling to unravel the underlying mechanisms of complex, heterogeneous psychiatric diseases, and provide evidence in support of oligodendrocyte precursor cell dysfunction as a novel candidate mechanism of schizophrenia.

METHODS SUMMARY

Genetic analysis

Linkage and Copy Number Analysis was performed with Illumina HumanCytoSNP-12v2 chip arrays using an affected-only model with an assumption of 99.9% penetrance. Analysis revealed a total of 294.34 Mb of genomic regions, with suggestive linkage on chromosomes 2, 11, 14, 15, and 16. Whole-genome exome sequencing was performed twice: initially at 40x, and again at 90x coverage. Exome variants were considered for additional validation if they were rare (MAF < 0.001), predicted to alter coding sequence (missense, nonsense, frameshift, essential splice site), and occurred within the regions of suggestive linkage.

Cellular studies

Human iPSCs were differentiated to NPCs and neurons by embryoid body (EB) based neural differentiation. Electrophysiology was performed in whole cell patch-clamp configuration after 8-10 weeks of differentiation. iPSC-derived OPCs were differentiated according to Monaco *et al*[66] with modifications. Biotinylation of cell surface proteins was adapted from Huang *et al*[67].

Magnetic Resonance Imaging (MRI)

Two patients and one control sibling of the family were subjected to MRI scanning. Population controls (n=294) matched on age, gender, and pack-years of cigarette smoking were selected from the Rotterdam Study. MRI images were obtained using a 1.5 Tesla General Electric (GE Healthcare, Milwaukee, Wisconsin, USA) MR system using a bilateral phased-array head coil. A full description of the imaging protocol and Rotterdam Study design has been described elsewhere[68]. An in-house MATLAB (Mathworks, Natick, MA) program was used to quantify the number and spatial characteristics of white matter 'potholes' along the major white matter tracts[69].

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Author contributions: F.M.dV., C.G.B., N.G., V.B. and S.A.K. designed the experiments. C.G.B. and S.A.K. established the family-based ascertainment program. C.G.B., F.M.dV. and M.C. conducted the family recruitment. C.G.B. and F.M.dV. performed the genetic analysis,

with assistance from M.Q., S.O. and G.J.B. B.D. conceived the protein misfolding hypothesis and implemented the structural modeling. D.C., R.M.K., P.R.B., A.N., A.T.S., J.H., E.H., V.Bergink, N.A., A.H., C.M.vD., H.T., A.U., J.A.V and GROUP Study Consortium provided case-control cohort samples. C.G.B., M.A.I. and M.W.V. coordinated the brain imaging studies. C.D.L. and T.W. performed the brain imaging data analyses. T.dW., F.W.V. and H.B.B. established the fibroblast lines. M.G., T.L. and J.G. performed the iPSC reprogramming. F.M.dV. and N.G. conducted the neuronal and oligodendrocyte progenitor cell experiments. G.S. performed the electrophysiological recordings. B.L. and M.B. assisted with the oligodendrocyte progenitor cell studies. Genetic analyses were supervised by V.B. and S.A.K. iPSC and brain imaging studies were supervised by S.A.K. F.M.dV., C.G.B., N.G. and S.A.K wrote the manuscript. The manuscript was reviewed, edited and approved by all authors.

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SUPPLEMENT

Content

Supplementary Tables

eTable 1
Discovery Family – Patient characteristics

Patient	II-1	III-5	III-7	III-9	III-11
Diagnosis (DSM)	SZ	SZ	SZ	SZ	SZ
Age of onset first psychosis	46	18	36	18	15
Medication	n/a	Clozapine Biperidene Lithium Oxazepam	Quetiapine Paroxetine Lorazepam	Zuclopenthixol	Zuclopenthixol Trihexyphenidyl Lithium Lorazepam

eTable 2.
Linkage analysis – Affected only model

Chr	Start position	End position	Start SNP	End SNP	Mb	Max LOD score
2	2686579	17262830	rs11694900	rs17476649	14.576251	0.8809
4	14744996	20524715	rs4464561	rs16869706	5.779719	0.8808
6	164665485	End	rs942731	rs9459964	5.675712	0.8809
7	Start	2739017	rs6583338	rs809547	2.692778	0.8852
7	69935641	97595184	rs4717530	rs9692345	27.659543	0.8852
7	148239179	End	rs6957883	rs1125769	10.809432	0.8852
10	1147045	9247586	rs4880763	rs1469993	8.100541	0.8808
11	Start	4905155	rs1045454	rs11603903	4.700927	0.8852
11	68289796	129160763	rs11228269	rs4644651	60.870967	0.8852
14	89036519	End	rs2116445	rs2583292	18.068524	0.8852
15	39135102	101779863	rs7167406	rs11858464	62.644761	0.8852
16	10480846	76592633	rs7195621	rs12599021	66.111787	0.8826
19	52447068	End	rs8105910	rs7910	6.646396	0.8851

eTable 3.
Discovery family candidate variants

Chr	Genomic Position (hg19)	REF	ALT	Gene symbol	Nr Unaffected	MAF ExAC
11	108014748	A	G	ACAT1	1	0.00042
11	113679159	T	C	USP28	1	3.0×10^{-5}
15	75983015	C	T	CSPG4	0	7.8×10^{-5}
16	23080205	C	T	USP31	2	1.6×10^{-5}
16	31371780	T	C	ITGAX	2	4.6×10^{-5}

eTable 4
Sanger sequencing primers for *CSPG4* open reading frame

PCR amplification primers

Name	Sequence
CSPG4_ex01_F	ctgccccagagaggaacagc
CSPG4_ex01_R	cccctaactggacagccttgg
CSPG4_ex02_F	gggctggacacaaggtgagc
CSPG4_ex02_R	caagagcctggcagcaagc
CSPG4_ex03a_F	tgccacagcctccaaagtagc
CSPG4_ex03a_R	gcagagtccgggtcataggc
CSPG4_ex03b_F	gctggaggtgtcggtgacg
CSPG4_ex03b_R	ggcacgtgcacacatgtaacc
CSPG4_ex04_F	accagctgcatgtctggctgc
CSPG4_ex04_R	ctggctccgaggagttgtgagg
CSPG4_ex05_F	cagtctgggggttatacacagagagg
CSPG4_ex05_R	gctctgagccgcgaagtagg
CSPG4_ex06-07_F	agctggggccttcctgggta
CSPG4_ex06-07_R	gccaggtccaggcctgtgttt
CSPG4_ex08_F	ggtcacgctgcctctttgc
CSPG4_ex08_R	acgtctgctgccagtgatgc
CSPG4_ex09_F	cccagagtggggcctgag
CSPG4_ex09_R	cccaaccatcaagccaggtc
CSPG4_ex10a_F	gggagggacaatgggagagg
CSPG4_ex10a_R	ccagctcgccagcatctagg
CSPG4_ex10b_F	ctccgggtggtttcagatcg
CSPG4_ex10b_R	tctccaggctcggagtgagc

Internal Sanger sequencing primers

Name	Sequence
CSPG4_ex3a_int_f1	atgcagccaccctcaatgg
CSPG4_ex3a_int_r1	tcctcctccagcctgcagc
CSPG4_ex3a_int_f2	cgtcacctccaggaacaccg
CSPG4_ex3a_int_r2	ggcagccagagagtgggg
CSPG4_ex3b_int_f1	ctggcccaaggctctgccat
CSPG4_ex3b_int_r1	ggtgccctggcctccttgag
CSPG4_ex3b_int_f2	acaaggctgtcagatggccagg
CSPG4_ex3b_int_f3	ggaggtacggggtgtcttccg
CSPG4_ex3b_int_f4	ccaacctcgacatccgcagtg
CSPG4_ex3b_int_r2	gccggccacgcaacagg
CSPG4_ex3b_int_r3	tgggtgttctgagtgtgcagtgg
CSPG4_ex3b_int_r4	cggcaggagaactcggtcg
CSPG4_ex10a_int_f1	cccagctggctgcagggc
CSPG4_ex10a_int_f2	catcgaggtgcagctgcggg
CSPG4_ex10a_int_r1	ctgccacgctgctcccgttg
CSPG4_ex10a_int_r2	ccggctggggaactgtgtgac
CSPG4_ex10b_int_f1	gaccttgaggacgggaggct
CSPG4_ex10b_int_f2	ctgactgccaagccccgcaa
CSPG4_ex10b_int_r1	gtaaggctcagtggcaaagtcca
CSPG4_ex10b_int_r2	atctaggacggtggggtccagg

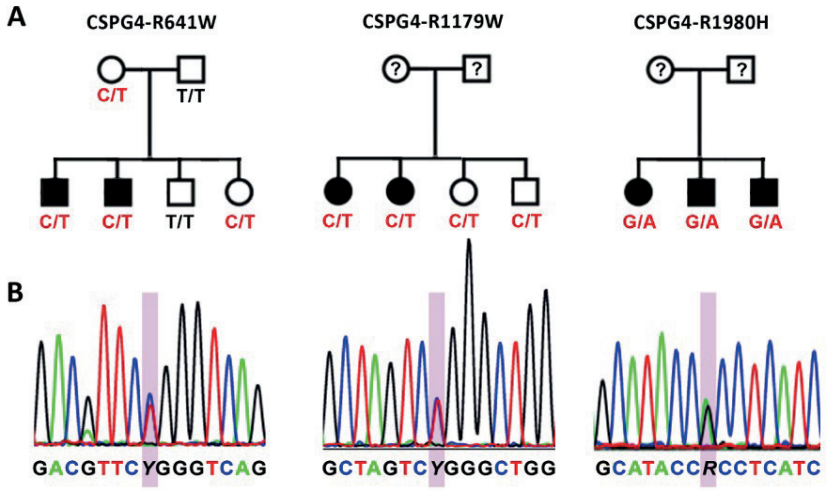
eTable 5.
qPCR primers for iPSC pluripotency genes

hOCT3/4-F	GAC AGG GGG AGG GGA GGA GCT AGG
hOCT3/4-R	CTT CCC TCC AAC CAG TTG CCC CAA AC
hSOX2-F	GGG AAA TGG GAG GGG TGC AAA AGA GG
hSOX2-R	TTG CGT GAG TGT GGA TGG GAT TGG TG
hNANOG-F	CAG CCC CGA TTC TTC CAC CAG TCC C
hNANOG-R	CGG AAG ATT CCC AGT CGG GTT CAC C
hGDF3-F	CTT ATG CTA CGT AAA GGA GCT GGG
hGDF3-R	GTG CCA ACC CAG GTC CCG GAA GTT
hREX1-F	CAG ATC CTA AAC AGC TCG CAG AAT
hREX1-R	GCG TAC GCA AAT TAA AGT CCA GA
hFGF4-F	CTA CAA CGC CTA CGA GTC CTA CA
hFGF4-R	GTT GCA CCA GAA AAG TCA GAG TTG
hESG1-F	ATA TCC CGC CGT GGG TGA AAG TTC
hESG1-R	ACT CAG CCA TGG ACT GGA GCA TCC
hTERT-F	CCT GCT CAA GCT GAC TCG ACA CCG TG
hTERT-R	GGA AAA GCT GGC CCT GGG GTG GAG C
hKLF4-F	TGA TTG TAG TGC TTT CTG GCT GGG CTC C
hKLF4-R	ACG ATC GTG GCC CCG GAA AAG GAC C
h-cMYC-F	GCG TCC TGG GAA GGG AGT TCC GGA GC
h-cMYC-R	TTG AGG GGC ATC GTC GCG GGA GGC TG

Supplementary Figures

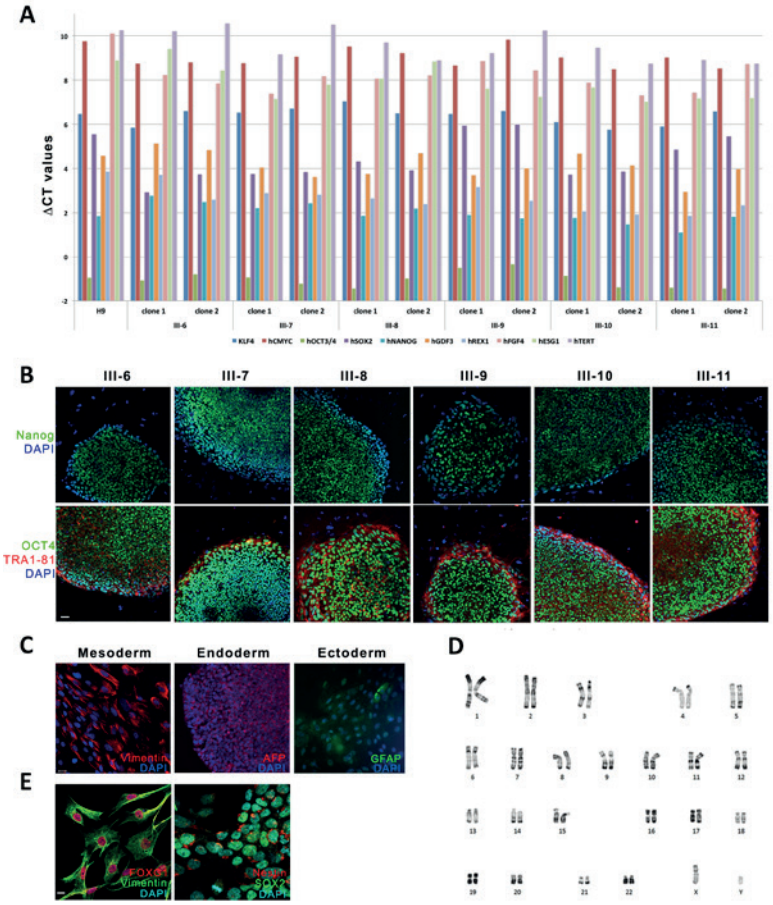
eFigure 1. Additional rare coding variants in *CSPG4*.

A: Pedigrees with *CSPG4*^{R641W}, *CSPG4*^{R1179W}, and *CSPG4*^{R1980H} variants. Symbols: filled, schizophrenia; open, unaffected; heterozygous carriers of the *CSPG4* c.1921C>T p.R641W, c.3535 C>T p.R1179W, and c.5939 G>A p.R1980H. **B:** Representative sequencing results for heterozygous carriers of the corresponding *CSPG4* mutations in panel A.



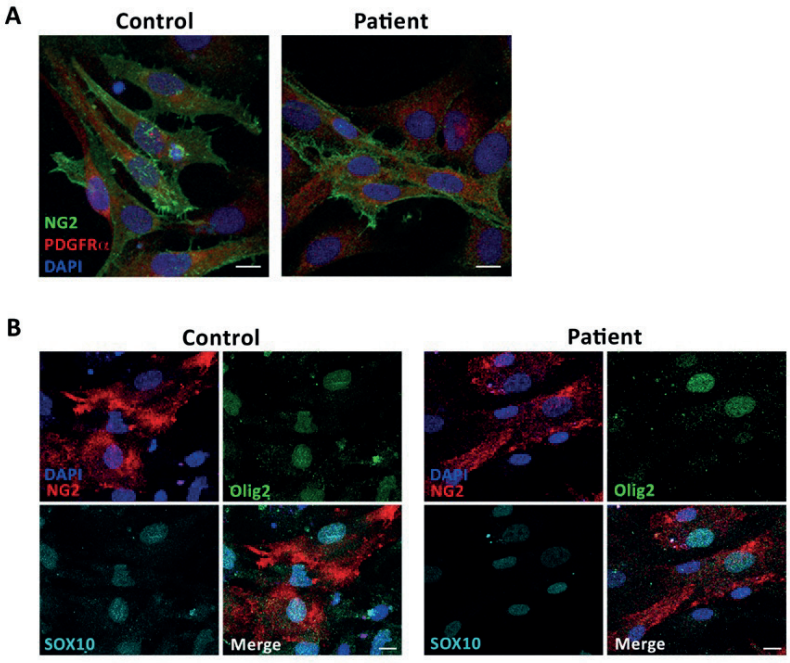
eFigure 2. Characterization of iPSC clones and NPCs.

A: RT-PCR of pluripotency genes normalized to GAPDH expression levels confirmed down-regulation of exogenous reprogramming genes and up-regulation of endogenous stem cell genes, similar to H9 human embryonic stem cells. **B:** iPSC colonies showed uniform staining for pluripotency markers Nanog, Oct4 and TRA1-81. **C:** EB differentiation confirmed that iPSCs were capable of generating representative cell types of all 3 embryonic layers – endoderm (AFP), mesoderm (Vimentin) and ectoderm (GFAP) (scale bar = 20 μm). **D:** Karyotyping was performed on all iPSC clones to confirm genomic integrity following reprogramming. Shown is a representative karyogram from the iPSC line of subject III-11. **E:** NPCs were positive for SOX2, Nestin, Vimentin and FOXG1, confirming their forebrain specification (scale bar = 10 μm).



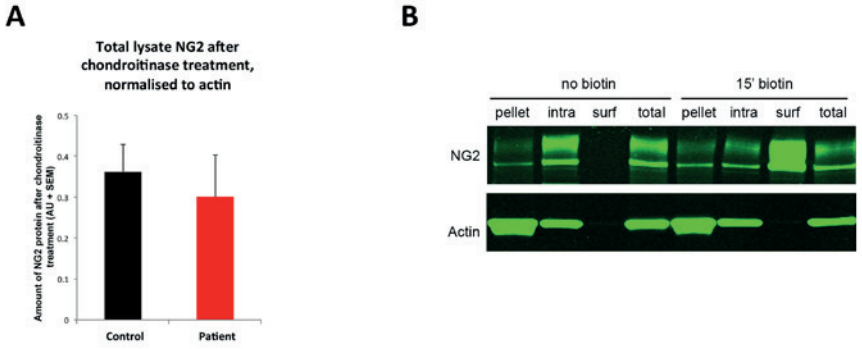
eFigure 3. Characterization of OPCs.

NG2+ cells co-expressed the OPC markers PDGFRα (**A**), Olig2 and SOX10 (**B**) (scale bars = 15 μm).



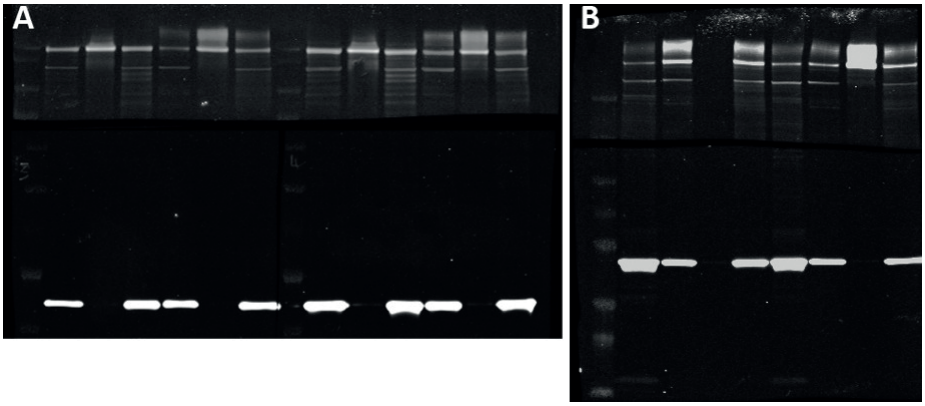
eFigure 4. Biotinylation assay controls.

A: Following chondroitinase ABC treatment, the abundance of 300 kDa (unmodified) NG2 in the whole cell lysate fraction is similar between patient and control OPCs (n=6 control and 6 patient clones, t=0.51, *P*=0.62). **B:** In the absence of biotin (left panel), NG2 protein is undetectable in the Neutravidin-purified surface fraction. Furthermore, the absence of detectable actin in the surface protein samples confirms the high specificity of the biotin purification.



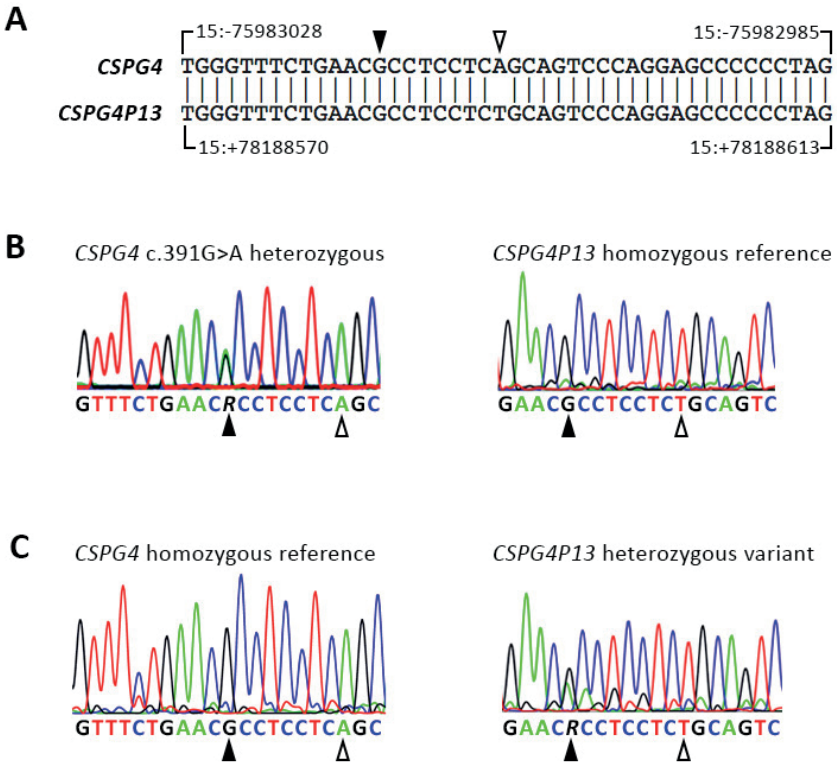
eFigure 5. Raw LI-COR western blot images.

Full western blots for NG2 protein (upper) and re-blotting for actin (below). **A**: Full blot corresponding to Figure 3D (lower half cut vertically for processing). **B**: Full blot corresponding to eFigure 4B.



eFigure 6. *CSPG4P13* pseudogene sequencing controls.

A: Partial alignment of *CSPG4* Taqman amplicon (hg19 chromosome 15: 75982916-75983072) with homologous *CSPG4P13* pseudogene sequence (hg19 chromosome 15: 78188526-78188682). Filled arrow indicates the position of the *CSPG4* c.391G>A variant. Open arrow indicates the differential nucleotide distinguishing the flanking *CSPG4* sequence from *CSPG4P13*. **B:** Sanger traces of an individual carrying the *CSPG4* c.391G>A variant while homozygous reference for *CSPG4P13*. **C:** Sanger traces of a sample called falsely positive by Taqman genotyping due to a homologous *CSPG4P13* polymorphism (genomic position, hg19 chromosome 15: 78188583) while being homozygous reference for *CSPG4* c.391G.



eMethods

Recruitment of family with schizophrenia and obtaining patient material

A Dutch white Caucasian non-consanguineous family with a high incidence of schizophrenia was ascertained. Written informed consent was obtained from all participating subjects. This study was approved by the medical ethical committee of the Erasmus University Medical Centre (Rotterdam, The Netherlands). Participating family members were screened for current or past psychiatric symptoms using the Structural Clinical Interview for DSM-IV (SCID-1)[1]. Medical screening revealed no evidence of somatic comorbidities, dysmorphologies, or neurological symptoms. All individuals with schizophrenia were documented to be of average intelligence at the time of their initial diagnosis. DNA isolation from venous blood samples and skin biopsies were performed using standard procedures. Two family members (II-2 and III-9) passed away since the start of the study.

For skin biopsies, a small area of skin of the medial aspect of the upper arm was anesthetised with an EMLA patch (AstraZeneca) for one hour, after which the skin was disinfected with ethanol and the biopsy was obtained through all skin layers with a standard 3mm biopsy punch. The tissue was collected in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-Invitrogen) without additives and transferred to culture within 24 hours. Primary human fibroblasts were cultured in DMEM containing 10% fetal calf serum and 1% penicillin/streptomycin (P/S).

Genetic analysis

Genomic DNA was isolated using standard methodology from 14 family members, including 13 by whole blood samples and one subject (pedigree ID II-1) using paraffin-embedded tissue (**Figure 1A**). Linkage and Copy Number Analysis was performed with Illumina HumanCytoSNP-12v2 chip arrays (294,975 markers). Linkage analysis was conducted exclusively for the purpose of identifying a genome-wide set of candidate chromosomal regions shared by all affected family members (defined as those regions with a LOD score > 0). Linkage was performed using Allegro and implemented in the EasyLinkage version 5.08 interface [2] with one marker every 0.5 cM using a co-dominant allele frequency algorithm and dominant scoring function. Unaffected family members were considered as having an unknown affection status in the linkage model, providing no *a priori* constraints on the rate of incomplete penetrance. Copy number analysis was performed using NEXUS discovery edition, version 6 (BioDiscovery, El Segundo, CA), which did not indicate the presence of copy number variants segregating with the phenotype. The strongest evidence of linkage was observed on chromosomes 2, 11, 14, 15, and 16, involving a total of 294.34 Mb of genomic loci.

Whole exome sequencing was performed on three individuals of the discovery family (pedigree IDs: II-2, III-5 and III-9). Sequencing was performed twice for all three samples: initially at 40x, and subsequently at 90x coverage. Exome sequencing was performed using in-solution capturing (Agilent SureSelect V2 and V4 Human 50 Mb kit respectively, Agilent Technologies) and paired-end sequencing with Illumina Hi-Seq 2000 sequencers. Reads were aligned to the human reference genome version 19 using Burrows-Wheeler Aligner. SNPs and indels were called using the Genome Analysis Toolkit (GATK).

The heterozygous variants were filtered based on the following criteria: a) present within the candidate genomic regions shared among all affected family members, b) predicted to affect protein coding (missense, nonsense, frameshift, splice site), c) called in at least one of the affected individuals [III-5 and III-9], d) absent from the unaffected mother [II-2], e) absent from dbSNP129, and f) with a minor allele frequency (MAF) of < 0.001 in in the Exome Aggregation Consortium (ExAc) browser (European non-Finnish)[3], Exome Variant Server

(EVS6500, NHLBI Exome Sequencing Project)[4], 1000 Genomes[5], and Genome of the Netherlands[6] cohorts.
Genotyping of *CSPG4* c.391G>A was performed in a cohort of Dutch subjects with 1219 schizophrenia cases and 10,611 controls. Genotyping of 763 schizophrenia cases and 386 healthy control subjects were performed using a custom Illumina Infinum Human Exome Beadchip array (Genetic Risk and Outcome of Psychosis [GROUP] / Utrecht cohort study[7]). TaqMan genotyping was performed on an additional 456 locally-collected schizophrenia cases and 10,225 population-based controls filtered for identity by descent ($\hat{\pi} < 0.25$) (Rotterdam Study[8]). All samples with ALT calls by exome array or TaqMan genotyping were validated by Sanger sequencing, particularly given the existence of the *CSPG4P13* pseudogene with high homology to the region surrounding *CSPG4* c.391G>A (**eFigure 6**).
Taqman genotyping of *CSPG4* c.391G>A was performed with the following primers and probes: F_GGCTGGTTCCCCTCAGGTA, R_GTGGTGCTGACTGTCGTAGAG, VIC_TTTCTGAACGCCTCCTC, FAM_TTTCTGAACACCTCCTC.
The following primer sets were used for Sanger sequencing of *CSPG4* c.391G>A: F_TCTGGGGCCCCAAGTGTGG and R_AGAGTGGGGCCCAGAGAAGC, with an internal forward primer for sequencing: Fseq_GGGCCAGGAGGAGCTGAGG. A second primer set was used for confirmation: F2_CCACTCCCCATCTCTCTTCAGG and R2_CAGGGCCACATCATCACTGG.
CSPG4P13 pseudogene-specific amplification was performed using the following primers: F_CTCTGGGGCACCAAGAGTGG and R_AGAGTGGGGCCCAGAGAAGC.

Structural homology modelling
Homology modeling of the first Laminin G domain (amino acids 29-176) was performed independently using both the Protein Homology/analogY Recognition Engine – Version 2.0 (Phyre2)[9] and Iterative Threading ASSEmbly Refinement (I-TASSER)[10] protein structure prediction servers. All 148 residues (100%) of the reference and mutant sequences were modeled at >99% confidence by Phyre2 using intensive mode. For I-TASSER, the confidence (C-score) was 0.81 and topological similarity (TM-score) was 0.82+/- 0.08, for both models. Reference and mutant models were structurally aligned with PyMol (http://www.pymol.org).

Generation and characterization of iPSCs
Reprogramming of human primary skin fibroblasts was performed as previously described[11]. Briefly, fibroblasts were infected with a multicistronic SIN lentiviral vector containing an SFFV promoter, encoding OCT4, SOX2, KLF4 and MYC, as well as dTomato to visualize reprogramming. Emerging iPSC colonies were cultured on γ-irradiated mouse embryonic feeder (MEF) cells.
Characterization of iPSC clones was performed by RT-PCR (**eFigure 2A**), immunostaining for un-differentiated human ES markers (**eFigure 2B**) and markers of three embryonal germ layers on embryoid bodies (EBs) differentiated *in vitro* (**eFigure 2C**). Total RNA of iPSCs was isolated for RT-PCR using standard protocols (primers listed in **eTable 5)**. For EB differentiation, iPSC colonies were dissociated by collagenase IV treatment and transferred to ultra-low attachment 6-well plates (Corning). Floating EBs were cultured in iPSC medium without bFGF for a minimum of 6 days with supplemented SB431542 (Tocris Bioscience) for ectoderm conditions only. EBs designated for endoderm differentiation were transferred to gelatin-coated 12-well plates containing the following medium: RPMI 1640 (Gibco-Invitrogen), supplemented with 20% FBS, 1% P/S, 1% glutamine and 0.4mM alpha-thioglycerol. Mesoderm differentiation from the EBs was induced in gelatin-coated 12-well-plates with DMEM medium (low glucose) supplemented with 15% fetal bovine serum, 1% P/S, 1% glutamine and 1% MEM-non-essential amino acids. Ectoderm differentiation was

induced in Matrigel (BD)-coated plates with the following medium: neurobasal medium (Gibco-Invitrogen) and DMEM/F12 (v/v 50/50) supplemented with 1% P/S, 1% glutamine, 1% MEM-non-essential amino acids, 0.02% BSA (Gibco-Invitrogen), 0,5% N2 (Gibco-Invitrogen) and 1% B27 (Gibco-Invitrogen). After two weeks in culture, cells were fixed with 4% formalin for immunolabeling.
iPSCs were cultured in standard ES cell culture medium containing DMEM/F12 (Gibco-Invitrogen) supplemented with 20% knock-out serum replacement (Gibco-Invitrogen), 2mM L-glutamine (Gibco-Invitrogen), 1% P/S (Gibco-Invitrogen), 1% MEM-non-essential amino acids (PAA Laboratories GmbH), 0.1mM β-mercapto-ethanol, and 10 ng/ml bFGF (Gibco-Invitrogen). Medium was replenished daily and colonies were passaged weekly using collagenase IV (1 mg/ml, Gibco-Invitrogen) with 10μM ROCK inhibitor (Y-27632, Sigma).

Karyotype analysis
iPSCs were dissociated to single-cell suspension using TrypLE Express (Gibco-Invitrogen) and plated feeder-free in mTeSR1 medium on three Matrigel-coated wells of a 6-well plate in the presence of 10μM ROCK inhibitor. The next day, cells were harvested using TrypLE Express, treated with colcemid (200 ng/ml) and hypotonic solution, and fixed using Carnoy's Fixative. At least 20 metaphases were analysed for each clone, for which the chromosome count was considered normal if more than 70% of cells analysed had 46 chromosomes. More detailed karyotypic analysis with RBA and QFQ band analysis was performed for one individual patient clone (individual III-11) to further exclude segregating cytogenetic abnormalities (**eFigure 2D**).

Neuronal differentiation
iPSC colonies were dissociated from MEFs with collagenase and transferred to non-adherent plates in hES cell medium on a shaker in an incubator at 37ºC/5% CO_2. After two days, EBs were changed to neural induction medium [DMEM/F12, 1% N2-supplement (Gibco-Invitrogen), 2 μg/ml heparin (Sigma), 1% P/S] and cultured for another four days in suspension. EBs were gently dissociated and plated onto laminin-coated dishes in neural induction medium. Cells were dissociated with collagenase after 8 days and plated onto laminin-coated dishes in NPC medium [DMEM/F12, 1% N2, 2% B27-RA, 1 μg/ml laminin (Sigma) and 20 ng/ml FGF_2 (Millipore), 1% P/S]. After one week, NPCs were dissociated with collagenase, re-plated, and passaged 1:4 weekly. For neural differentiation, passage 5 NPCs were plated on coverslips coated with 100 μg/ml PDL (Sigma) and 50 μg/ml laminin in neural differentiation medium consisting of Neurobasal medium supplemented with 1% MEM-non-essential amino acids, 1% N2 supplement, 2% B27-RA supplement, 20 ng/ml BDNF (ProSpecBio), 20 ng/ml GDNF (ProSpecBio), 1 μM db-cAMP (Gibco-Invitrogen), 200 μM ascorbic acid (Gibco-Invitrogen), 2 μg/ml laminin and 1% P/S.

Electrophysiological recordings
After 8-10 weeks of neuronal differentiation, culture slides were transferred to the recording chamber following a thirty-minute serial partial exchange of cell culture medium with artificial cerebrospinal fluid (ACSF) containing the following (in mM): 110 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 0.2 ascorbate (pH 7.4). In the recording chamber, slides were continuously perfused with ACSF at 1.5-2 mL/min, saturated with 95% O_2/5% CO_2 and maintained at 20-22°C.
Whole-cell patch-clamp recordings were performed under infrared differential interference contrast visual guidance using an upright microscope (Zeiss) with borosilicate glass recording micropipettes (3-6 MΩ) filled with the following medium (in mM): 130 K-gluconate, 11 KCl, 10 HEPES, 5 NaCl, 0.1 EGTA, 1 $MgCl_2$, 2 Mg-ATP, 0.3 Na-GTP, 5 phosphocreatine (pH

7.4). Data were acquired at 10 kHz using an Axon Multiclamp 700B amplifier (Molecular Devices), filtered at 3 kHz, and analyzed using pClamp 10.1 (Molecular Devices). Whole-cell capacitance and series resistance were compensated, and voltage was adjusted for liquid junction potential.
Current-clamp recordings were performed at a holding potential of -60 mV. Passive membrane properties were analysed using a series of hyperpolarizing and depolarizing square wave currents (500 msec duration, 1 sec interstimulus interval) in 5 pA steps, ranging from -20 to +30 pA. AP amplitude, rise time, decay time and half width were measured for the first evoked AP resulted by a depolarizing step, from the threshold to the peak, for which the threshold was defined by the moment at which the second derivative of the voltage exceeded the baseline. Spontaneous APs were recorded at resting membrane potential. Voltage-clamp recordings were performed at a holding potential of -90 mV.

Oligodendrocyte lineage differentiation
iPSC-derived NPCs were differentiated to OPCs according to Monaco *et al*[12], with modifications. NPCs were plated on laminin-coated 10 cm plates in NPC medium consisting of DMEM/F12, 1% N2, 2% B27-RA, 1mg/ml laminin, 1% P/S, 25 ng/ml basic Fibroblast Growth Factor (bFGF) and 20 ng/ml Epidermal Growth Factor (EGF).
When NPCs reached 90% confluence, NPC medium was changed to OPC differentiation medium for three weeks: DMEM/HAMS F12, 1% N2, 1% BSA, 1% L-Glutamine, 1% P/S, 20 ng/ml bFGF, 10 ng/ml platelet derived growth factor (PDGF-AA, ProspecBio), 2ng/ml Sonic hedgehog (Shh), 2 ng/ml neurotrophic factor 3 (NT-3) and 3 nM triiodothyronine (T3). OPC medium was changed every other day, and cells were passaged weekly at 1:4.

Surface Biotinylation
Biotinylation of cell surface proteins was adapted from Huang *et al*[13], with modifications. First, the OPCs were washed twice in cold PBS/CaCL2/MgCl2 (2.5 mM CaCl2, 1 mM MgCl2, pH 7.4). Then, the OPCs were incubated for 15 minutes on ice with or without 0.3mg/ml of freshly dissolved Sulfo-NHS-SS-Biotin (Thermo Scientific) in PBS/CaCL2/MgCl2. Next, the OPCs were washed three times on ice in cold biotin quenching solution consisting of 50 mM glycine in PBS/CaCl2/MgCl2. Subsequently, the OPCs were lysed by incubation for 10 minutes on ice with lysis buffer, pH7.4, consisting of 5mM EDTA, 5mM EGTA, 1% Phosphatase inhibitor cocktail 2 and 3 (Sigma), 1% PIC (Sigma), and 1% Triton X in PBS. At least 3 wells were pooled from each cell line at each experimental condition, to minimize any plating or growing variability in the cell culture. A portion of the lysate was removed and labeled as the total fraction (whole cell lysate). The remaining lysate was then centrifuged (13200 rpm at 4°C for 15 minutes) to remove insoluble proteins. The supernatants were incubated overnight at 4°C with PBS-washed and lysis buffer-equilibrated Pierce™ NeutrAvidin™ Agarose beads (Life Technologies). Next, the mixture was spun down at 2000 rpm, separating the supernatant termed the intracellular fraction from the surface protein fraction attached to the beads. Subsequently, the beads were washed four times by rotating with lysis buffer and after the final wash the beads were dried completely and taken up in 20µl of lysis buffer, labeled as the surface fracion.

Sodium dodecyl sulfate (SDS)-PAGE and Western Blotting:
First, 25% XT Sample Buffer (Biorad) and 10% 100mM dithiothreitol (DTT) were added to equal volumes of cell extract followed by heating to 65°C for 15 minutes. Then, the samples were electrophoresed through 4%-12% Criterion™ XT Bis-Tris gradient gels (Biorad) in XT-Mops buffer (Biorad). Proteins were transferred overnight at 4°C on 0.45µm pore

nitrocellulose membrane (Biorad) in a Tris-Glycine buffer consisting of 10% Tris/Glycine buffer (Biorad) and 20% anhydrous methanol in distilled water. Membranes were blocked for 2 hours while shaking at room temperature in TBST buffer containing 4% blotting grade blocker (Biorad). Then, the membranes were incubated with a monoclonal antibody generated from hybridoma B5 cells raised against purified intact NG2 protein from melanoma cells (generous gift from W.B. Stallcup) and mouse anti-actin (Sigma) in TBST buffer containing 1% Tween and 2% milk for 48 hours at 4°C followed by washing with TBST buffer. Finally, the blots were incubated with IRDye® secondary antibodies (LI-COR) against the primary antibody species for two hours at room temperature. The blots were washed twice in TBST and TBS followed by one wash in water, subsequently the antibody fluorescence was visualized using an ODYSSEY® CLx scanner (LI-COR). Quantification of NG2 western blot bands was performed using ImageJ software (http://imagej.nih.gov/ij/).

Immunocytochemistry and imaging

Cell cultures were fixed using 4% formalin in PBS. Primary antibodies were incubated overnight at 4°C in labeling buffer containing 0.05 M Tris, 0.9% NaCl, 0.25% gelatin, and 0.5% Triton-X-100 (pH 7.4). The following primary antibodies were used: SOX2, Nestin, MAP2, NeuN, GFAP, FOXG1 [ProSci], NG2-EC [kindly provided by W.B. Stallcup], Olig2 [Abcam], Vimentin [Santa Cruz Biotechnology], AFP [R&D Systems], TRA-1-81, Nanog [Beckton Dickinson], Oct4 [Abcam], Calreticulin [Fisher Scientific], SOX10 [R&D Systems]. The following secondary antibodies were used: Alexa-488, Alexa-546, Alexa-555 and Cy3 antibodies [Jackson ImmunoResearch]. Samples were imbedded in Mowiol 4-88 (Fluka) after which confocal imaging was performed with a Zeiss LSM700 confocal microscope (Apochromatic 40x objective, 1.3 NA, oil immersion) using ZEN software (Zeiss, Germany). OPC area (μm^2) was quantified on maximum projection of z-stack images covering the full depth of each measured cell using a manually drawn surface contour in ImageJ.

Magnetic Resonance Imaging (MRI)

Two patients and two control siblings of the discovery family provided additional written informed consent for MRI scanning. One of the unaffected siblings was excluded due to a history of systemic chemotherapy known to influence white matter integrity [14]. Population controls (n=294) were selected from the Rotterdam Study based on matching for age, gender, smoking behaviour, and alcohol use. All subjects, including population controls and family members, were imaged using the identical MRI scanner and acquisition protocol.

MRI images were obtained using a 1.5 Tesla General Electric scanner (GE Healthcare, Milwaukee, Wisconsin, USA, software version 11X), with a bilateral phased-array head coil. A full description of the imaging protocol has been described previously [15]. Diffusion-weighted imaging was performed using echo planar imaging (EPI) sequences collected in 25 directions with a b value of 1,000 s/mm^2 and three b = 0 images. Additional sequence parameters were TR = 8,000 ms, TE = 74.6 ms, bandwidth 14.71 kHz, flip angle = 13, acceleration of 2, and a voxel resolution of 3.3 x 2.2 x 3.5 mm^3.

Pre-processing of the diffusion-weighted images was performed using FSL[16]. After conversion from dicom to nifti format, individual images were eddy-current corrected using FMRIB's eddy_correct[16] followed by skull stripping using BET[17]. Fractional anisotropy (FA) images were created by fitting a tensor model to the diffusion data using FMRIB's Diffusion Toolbox (FDT)[16]. All subjects' FA data were then aligned into common space using the nonlinear registration tool FNIRT[18], which employs a b-spline representation of the registration warp field [19]. Whole brain mean FA was calculated by masking each image using the Johns Hopkins University White Matter Atlas (JHU WMA)[20] and determining the mean intensity of FA within the mask.

An in-house MATLAB (Mathworks, Natick, MA) algorithm was used to quantify the number and spatial characteristics of white matter 'potholes' along the major white matter tracts[21]. The input to the algorithm was the set of FA images that had undergone non-linear registration into MNI space using TBSS[22]. No spatial filtering was applied to the images. The first step was to generate a voxel-by-voxel mean and standard deviation (SD) image of the 294 matched population controls. These group and SD images were then used to individually create a voxel-wide z-image for all of the subjects, including the three family members. This resulted in individual z-images with each voxel based on the mean and SD of the matched population controls. To ensure the search involved only white matter regions, each image was masked with the cortical areas defined by the Johns Hopkins University white matter atlas[20].
The individual z-FA images were then used to search for contiguous voxels of white matter below a threshold of $z < -2$[21,23]. Clusters were determined by thresholding each image and labeling contiguous voxels in three-dimensional space that fell below the defined threshold. Only clusters greater than 250 voxels were used in the analyses.

Statistical Analysis
Significance of observations was established for genetic case-control analyses using the Fisher's exact test. Brain imaging results in family members were evaluated using z-scores based on the matched population control cohort distribution. For functional studies, statistical comparisons were performed using a two-tailed Student's t-test or Kolmogorov-Smirnov test as indicated. Data are expressed as mean ± S.E.M, unless otherwise specified. The threshold for significance was set at $P<0.05$ for all statistical comparisons.

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Supplementary author information

Genetic Risk and Outcome of Psychosis (GROUP) investigators
R. Bruggeman, MD, PhD, Department of Psychiatry, University Medical Center Groningen, University of Groningen; W. Cahn, MD, PhD, Department of Psychiatry, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht; L. de Haan, MD, PhD, Department of Psychiatry, Academic Medical Center, University of Amsterdam; R. S. Kahn, MD, PhD, Department of Psychiatry, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, Utrecht, the Netherlands; C. Meijer, PhD, Department of Psychiatry, Academic Medical Center, University of Amsterdam; I. Myin-Germeys, PhD, South Limburg Mental Health Research and Teaching Network, EURON, Maastricht University Medical Center; J. van Os, MD, PhD, South Limburg Mental Health Research and Teaching Network, EURON, Maastricht University Medical Center, Maastricht, the Netherlands, and King's College London, King's Health Partners, Department of Psychosis Studies, Institute of Psychiatry, London, England; and A. A. Bartels-Velthuis, PhD, Department of Psychiatry, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands.

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A heterozygous loss-of-function variant in *GRM2* segregating in a family with bipolar disorder

Christian G. Bouwkamp[1,2,*], Geeske M. van Woerden[3,*], Hilde Lavreysen[4], Ineke Maaskant[1], Annet T. Spijker[5], P.M. Judith Haffmans[5,6], Erik Hoencamp[5,6], Femke M. de Vrij[1], Vincenzo Bonifati[2], Ype Elgersma[3], Steven A. Kushner[1]

1 Department of Psychiatry, Erasmus University Medical Centre, Rotterdam, The Netherlands
2 Department of Clinical Genetics, Erasmus University Medical Centre, Rotterdam, The Netherlands
3 Department of Neuroscience, Erasmus University Medical Centre, Rotterdam, The Netherlands
4 Neuroscience, Janssen Research and Development, Beerse, Belgium
5 PsyQ, Parnassia Psycho-Medical Centre, The Hague, the Netherlands
6 Leiden University, Faculty of Behavioural Sciences, Institute of Psychology
* These authors contributed equally

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Abstract

Background: Bipolar disorder is a severe psychiatric illness with a strong hereditary component. The molecular genetic underpinnings however remain largely elusive. Family-based genetic studies can provide biological insight into the aetiology of this disorder.

Methods: We performed linkage analysis coupled with exome sequencing in a family with bipolar disorder. Candidate-variants were confirmed by Sanger sequencing. We performed Sanger sequencing of the open reading frame (ORF) and exon-intron boundaries of the top-candidate, *GRM2,* in a cohort of Dutch patients with bipolar disorder. Functional work consisted of overexpression experiments of variant and reference alleles in dissociated mouse hippocampal neurons, in-vivo migration experiments in mouse hippocampus as well as radioactive binding studies in Chinese hamster ovary (CHO) cells, and fluorescent intensity analyses of membrane expression of the mGluR2 receptor.

Results: We identified a rare heterozygous missense variant (p.R750H) in *GRM2* segregating with bipolar disorder in the family we studied. Two additional rare missense variants in this gene were identified in a Dutch cohort of patients with bipolar disorder. Preliminary functional evidence indicates a possible loss-of-function mechanism for the R750H variant, which is in line with existing evidence regarding the involvement of mGluR2/3 signalling in psychosis.

Conclusions: We present genetic and functional evidence implicating the gene *GRM2* and its encoded protein mGluR2 in the aetiology of bipolar disorder.

Introduction

Bipolar disorder is a disabling psychiatric illness characterized by episodes of strongly elevated mood (mania), which may include psychotic features, and periods of depressed mood. The severe form of bipolar disorder (bipolar I disorder) affects ~1 percent of the population.[1] The treatment of bipolar disorder is mainly pharmacological, and is based on the use of mood stabilizers, antipsychotics, antidepressants, and benzodiazepines. In many instances, adjuvant psychotherapeutic interventions are indicated as well.

Bipolar disorder has a strong heritable component. From twin-based genetic studies, as well as single nucleotide polymorphisms (SNP) array-based heritability estimates, it is known that bipolar disorder has a heritability of ~0.7-0.8. This is comparable to schizophrenia and autism spectrum disorders (ASD). Together, they comprise the group of psychiatric disorders with the highest heritability.[2,3]

In the past decades, many families with a high incidence of bipolar disorder have been studied in order to identify the molecular genetic determinants and underlying molecular mechanisms. Many studies have been performed, ranging from candidate gene sequencing to family-based linkage, and genome-wide association studies (GWAS). These approaches yielded a vast array of different findings. The candidate-gene approach has been largely abandoned by the GWAS approach.[4] The findings from family-based linkage studies as well as GWAS studies are indicative of a number of genetic variants with high or low odds ratios, which are genuinely involved in the disease aetiology.

The advent of next generation sequencing (NGS) technology that allows for reading of all protein-coding nucleotides of the genome (the exome), or the entire genome, has revolutionized the field of human molecular genetics. In combination with family-based linkage[5], this has lead to the identification of many novel variants underlying rare Mendelian forms of human disease.[6]

Given the overlap of symptoms between bipolar disorder and schizophrenia, it is possible that there are pleiotropic effects of shared genetic factors which may result in either bipolar disorder or schizophrenia. Dysregulation of synaptic transmission mechanisms which have

been found in both schizophrenia and bipolar disorder might also be a mechanism which is shared across disease categories.[7]

Here, we describe the genetic analyses and functional work in a family with a high incidence of bipolar disorder. We performed linkage analysis coupled with exome sequencing in an effort to identify rare coding variants segregating with disease in the family. The top candidate gene was followed-up by Sanger-based validation in a cohort of Dutch patients with bipolar disorder. Furthermore, in order to assess the functional effect of the variant in the top candidate gene, we performed overexpression experiments in dissociated primary mouse neurons, as well as neuronal migration experiments in mice.

Methods

Family Ascertainment

A Dutch white Caucasian family was ascertained (**Figure 1A**). Diagnoses were made by SCID I interview as well as a review of their medical records where possible. Venous blood was drawn from all participating family members for DNA isolation according to standard protocols. The Erasmus University Medical Centre medical-ethical committee approved this research project and informed consent was obtained from all participants.

Genetic analyses

Linkage and copy number variant (CNV) analysis was performed with Illumina HumanOmniExpress 700k SNP-arrays on DNA isolated from venous blood. Linkage analysis was performed using an autosomal dominant affected-only model with an assumption that the pathogenic allele was inherited through the unaffected mother (Ped ID II-2). Linkage analysis was conducted for the purpose of identifying the genome-wide set of chromosomal regions shared by all affected family members Analysis revealed a total of ~840 Mb of shared genomic region (**Supplementary Table 1**). Linkage analysis was performed using Allegro embedded in EASYlinkage.[8] CNV analysis was performed using NEXUS discovery edition, version 7 (BioDiscovery, El Segundo, CA). Whole exome sequencing was performed on two affected siblings and their unaffected father (Ped IDs II-1 and III-1 and III-2) at 90x average coverage. Exome sequencing was performed using in-solution capture (Agilent SureSelect V4 Human 50 Mb kit, Agilent Technologies) and paired-end sequencing on an Illumina Hi-Seq 2000 sequencer at LGC Berlin. Reads were aligned to the human reference genome version 19 using

Burrows-Wheeler Aligner. SNPs and indels were called using the Genome Analysis Toolkit (GATK). Filtering of the variants was performed using Cartagenia software (Cartagenia Bench lab, Agilent Technologies). The heterozygous variants were filtered based on the following criteria: a) present within the shared genomic regions, b) predicted to affect protein coding (missense, nonsense, frameshift, splice site), c) called in both affected individuals (Ped IDs III-1 and III-2) and absent from their unaffected father (Ped ID II-1), d) absent from dbSNP129, and e) with a minor allele frequency (MAF) of < 0.1% in the more recent public databases (1000G, ExAC, GoNL). The remaining variants were genotyped by Sanger sequencing of all participating family members. We also performed Sanger sequencing of the open reading frame (ORF) and exon-intron boundaries of *GRM2* in a cohort of Dutch patients with bipolar disorder (for primers, see **Supplementary Table 2**).

Functional assays – neuronal transfection and in-utero electroporation

Constructs. The cDNA sequence from human *GRM2*-WT (NM_000839) was obtained from a human brain cDNA library by PCR (Phusion high fidelity, Thermo Fisher) using the following primers: Fw 5' GGCGCGCCACCATGGGATCGCTGCTTGCGCTCCTGGC 3' and Rev 5' TTAATTAATTATCAAAGCGATGACGTTGTCGAGTCC 3' and cloned into our dual promoter expression vector. The c.2549 G>A point mutation was introduced with site-directed Mutagenesis (Invitrogen) using the following primers: Fw 5' GCCTTCAAGACTCACAAGTGCCCCGAA 3' and Rev 5' TTCGGGGCACTTGTGAGTCTTGAAGGC 3'. The dual promoter expression vector was generated from the pCMV-*tdTomato* vector (Clontech), where the *CMV* promoter was replaced with a *CAGG* promoter followed by a multiple cloning site (MCS) and transcription terminator sequence. To assure expression of the tdTOMATO independent from the gene of interest, a PGK promoter was inserted in front of the *tdTomato* sequence. For all the *in vivo* and *in vitro* experiments, the vector without a gene inserted in the MCS was taken along as control (control vector) (**Figure 2A**).

Mice. For the neuronal cultures, FvB/NHsD females were crossed with FvB/NHsD males (both ordered at 8-10 weeks old from Envigo). For the *in utero* electroporation female FvB/NHsD (Envigo) were crossed with male C57Bl6/J (ordered at 8-10 weeks old from Charles River). All mice were kept group-housed in IVC cages (Sealsafe 1145T, Tecniplast) with bedding material (Lignocel BK 8/15 from Rettenmayer) on a 12/12 h light/dark cycle in 21°C (±1°C), humidity at 40-70% and with food pellets (801727CRM(P)) from Special Dietary

Service) and water available *ad libitum*. All animal experiments were approved by the Local Animal Experimentation Ethical Committee, in accordance with Institutional Animal Care and Use Committee guidelines.

Primary hippocampal cultures. Primary hippocampal neuronal cultures were prepared from FvB/NHsD wild type mice according to the procedure described in [9]. Briefly, hippocampi were isolated from brains of E16.5 embryos and collected altogether in 10 ml of neurobasal medium (NB, Gibco) on ice. After two washings with NB, the samples were incubated in pre-warmed trypsin/EDTA solution (Invitrogen) at 37° for 20 minutes. After 2 times washing in pre-warmed NB, the cells were resuspended in 1.5 ml NB medium supplemented with 2% B27, 1% penicillin/streptomycin and 1% glutamax (Invitrogen), and dissociated using a 5 ml pipette. Following dissociation, neurons were plated in a small drop on poly-D-lysine (25 mg/ml, Sigma) coated 15 mm glass coverslips at a density of $1*10^6$ cells per coverslip in 12 well plates containing 1 ml of supplemented NB for each coverslip. The plates were stored at 37°/5% CO_2 until the day of the transfection.

Neuronal transfection and immunocytochemistry. Neurons were transfected at different days *in vitro* (DIV) with the following DNA constructs: control vector (1.8 ug per coverslip), *GRM2*-WT and *GRM2*p.R750H (all 2.5 ug per coverslip). Neurons were transfected at DIV3, DIV7 and DIV14. Transfection of the plasmids was done using Lipofectamine 2000 according to the manufacturer instructions (Invitrogen). For the neuronal morphology analysis, neurons were fixed 5 days post-transfection with 4% paraformaldehyde (PFA)/4% sucrose and incubated overnight at 4°C with primary antibodies in GDB buffer (0.2% BSA, 0.8 M NaCl, 0.5% Triton X-100, 30mM phosphate buffer, pH7.4). For the membrane insertion analysis, transfection and fixation was done as for the neuronal morphology, but now neurons were incubated overnight with primary antibody either in PBS (membrane inserted GRM2 staining only) or GDB (global GRM2 staining) at 4°C. The following primary antibodies were used: guinea-pig anti MAP2 (1:500, Synaptic System) to stain for dendrites, n-terminal mouse anti mGluR2 (ab15672, Abcam). Secondary antibodies used were anti-mouse-Alexa488-, and donkey anti-guinea-pig-Alexa647-conjugated antibodies (all 1:200, Jackson ImmunoResearch). Slides were mounted using mowiol-DABCO mounting medium. Confocal images were acquired using a LSM700 confocal microscope (Zeiss).

For the analysis of the neuronal transfections, at least ten confocal images (20X objective, 0.5 zoom, 1024x1024 pixels) of different transfected neurons (identified by the red staining) were taken from each coverslip for each experiment with at least two independent replications. For

the analysis of the neuronal morphology, the NeuronJ plugin for ImageJ software was used to trace the dendrites with their branches. Total dendrite length, number of primary dendrites and number of branches were analysed. All the values were normalized against the mean value for each parameter of the control (control vector).

In utero electroporation. The procedure was performed in pregnant FvB/NHsD mice at E14.5 of gestation to target mainly the progenitor cells giving rise to pyramidal cells of the layer 2/3.[10,11] The DNA construct (1.5-3 ug/ul) was diluted in fast green (0.05%) and injected in the lateral ventricle of the embryos while still *in uterus*, using a glass pipette controlled by a Picospritzer® III device. To ensure the proper electroporation of the injected DNA constructs (1-2 ul) into the progenitor cells, five electrical square pulses of 45V with a duration of 50 ms per pulse and 150 ms inter-pulse interval were delivered using tweezer-type electrodes connected to a pulse generator (ECM 830, BTX Harvard Appartus). The electrodes were placed in such a way that the positive pole was targeting the developing somatosensory cortex. The following plasmids were injected: control vector, *GRM2*-WT and *GRM2*p.R750H. After birth, pups were sacrificed at P0 or P7 for histochemical processing (to look at neuronal migration).

Immunohistochemistry. Mice were deeply anesthetized with an overdose of Nembutal and transcardially perfused with 4% paraformaldehyde (PFA). Brains were extracted and post-fixed in 4% PFA. Brains were then embedded in gelatin and cryoprotected in 30% sucrose in 0.1M PB, frozen on dry ice, and sectioned using a freezing microtome (40/50 μm thick). Free-floating coronal sections were washed in 0.1M PB and a few selected sections were counterstained with 4′,6-diamidino-2-phenylindole solution (DAPI, 1:10000, Invitrogen) before being mounted with mowiol on glass. Overview images of the coronal sections were acquired by tile scan imaging using a LSM700 confocal microscope (Zeiss) with a 10X objective. Zoom in images of the targeted area were taken using a 20X objective.

Statistical analysis. Statistical difference between the conditions for the in vitro experiments was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons.

Functional experiments – radioligand binding experiments and membrane expression analysis in CHO cells

*GRM2*_R750H mutant and wildtype *GRM2* cDNA prepared by GeneArt® (Life Technologies) were transfected in CHO cells using Lipofectamine®. 72 hours after transient transfection, membrane preparation were made as previously described.[12] Radioligand binding was

performed using [3H]LY341495, an orthosteric mGlu2/3 antagonist.[12,13] A [35S]GTPγS binding assay was performed as described previously.[12] In each experiment, wildtype mGluR2 was assessed in parallel with the *GRM2*_R750H mutant, as well as a CHO cell line stably expressing *GRM2* and selected based on high mGluR2 expression. Western blot experiments were performed with the Ab 15672 antibody (Abcam, Cambridge, UK) against mGluR2 in order to assess mGluR2 membrane expression.

Results

Clinical Studies

The nuclear family consisted of an unaffected father and an unaffected mother who had three children affected with either bipolar I disorder (Ped IDs III-1 and III-2) or schizophrenia with depressive disorder and panic disorder (Ped ID III-3). Their affected daughter (Ped ID III-1) had two young daughters who did not have bipolar symptoms at the time of ascertainment (**Table 1**). From the mother's side, the family was heavily loaded with bipolar disorder in most generations (**Figure 1A**). Reduced penetrance was apparent as many asymptomatic family members had symptomatic offspring. Unfortunately, none of the affected family members in the extended family was available for study.

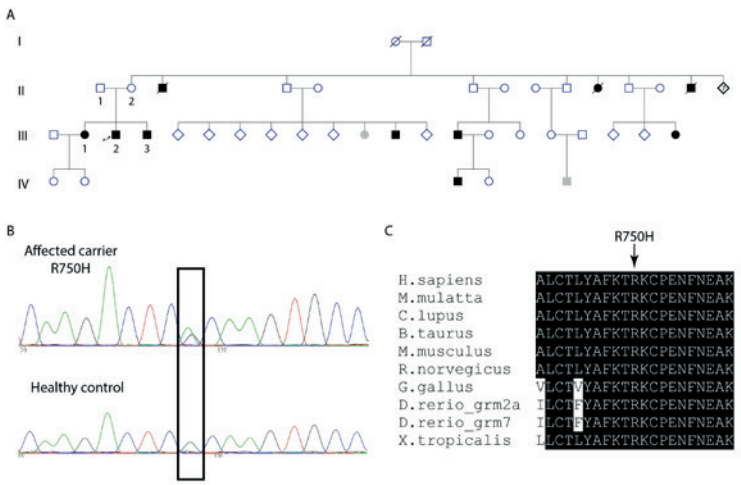


Figure 1 Pedigree and GRM2 variant

A) Family pedigree. Shaded symbols indicate family members. Subjects of whom DNA was available are numbered. Males are represented with squares and females with circles. **B)** Electropherograms indicating the heterozygous *GRM2* variant (affected family members) and the reference sequence (unaffected, unrelated subject). **C)** Amino acid conservation within the mGluR2 protein homologs across species.

Copy number variant analysis and parametric linkage analysis

Analysis of copy number variants with the NEXUS 7 software package did not reveal copy number variants segregating with disease. Parametric linkage analysis under an autosomal dominant model yielded ~840 Mb of shared genomic regions (**Supplementary Table 1**). These regions were followed-up with exome sequencing.

Exome sequencing

Analysis of the exome sequencing in combination with Sanger confirmation in all available DNA samples from the family revealed 14 segregating heterozygous variants (**Table 2**). Based on functional predictions scores and the ExAC probability of missense and loss-of-function intolerance scores (which is a statistical calculation based on the number of predicted and observed variants of each class in the gene), we selected the heterozygous c.2249G>A p.R750H variant in *GRM2*, which was present in all available affected family members and present in their unaffected mother who was assumed to be an obligate carrier of the variant (**Table 1 and Figure 1B**). In the largest public database to date, the Broad ExAC database, the minor allele frequency of this variant is 1/121390 (MAF $8.238*10^{-6}$). Furthermore, the amino acid sequence is highly evolutionarily conserved (**Figure 1C**).

We performed Sanger sequencing of the open reading frame and exon-intron boundaries of *GRM2* in 90 Dutch patients with bipolar disorder. This work revealed two rare coding variants (c.1073G>A p.R358Q, DbSNP ID rs148285622 and c.1571C>T p.P524L, DbSNP ID rs202090485) which were both located in the large extracellular domain of mGluR2. Family members of these patients were unfortunately not available for clinical assessment and segregation analysis.

Functional assays

The results of the neuronal transfection experiments at three days in-vitro (DIV3) indicate that in neurons transfected with the wildtype *GRM2* construct, the total dendrite length was increased compared to the neurons transfected with the control vector while in neurons transfected with the *GRM2*_R750H construct however, this effect was decreased (one-way ANOVA, $p < 0.002$, $F(2, 28) = 8.201$; empty vector vs WT *GRM2*: $p = 0.0004$; *GRM2*_R750H vs. WT *GRM2*: $p = 0.042$; *GRM2*_R750H vs. empty vector: $p= 0.072$) (**Figure 2B, upper panel**). This was also the case with respect to the level of complexity of dendrite

arborisation, where the arborisation was more complex in the neurons transfected with wildtype *GRM2* compared to the neurons transfected with the empty vector, while in neurons transfected with the *GRM2*_R750H mutant construct, this effect was decreased (one-way ANOVA, $p < 0.005$, $F(2, 28) = 10.047$; empty vector vs WT *GRM2*: $p = 0.0001$; *GRM2*_R750H vs. WT *GRM2*: $p = 0.055$; *GRM2*_R750H vs. empty vector: $p= 0.0218$) (**Figure 2B, upper panel**). After 7 days of differentiation, only the difference in arborisation complexity remained (one-way ANOVA, $p = 0.0055$, $F(2, 33) = 10.047$; empty vector vs WT *GRM2*: $p = 0.014$; *GRM2*_R750H vs. WT *GRM2*: $p = 0.1127$; *GRM2*_R750H vs. empty vector: $p= 0.0891$) (**Figure 2B, bottom panel**).

We next tested both the effect of mGluR2 wild type and mutant overexpression of the mGluR2 variant on neuronal migration. The migration patterns were analyzed in newborn mice at P0 and at P7. The preliminary experiments showed no clear difference between the wild-type and the mutated constructs (**Supplementary Figure 1A and B**). *In utero* electroporation of *GRM2* shRNA and imaging at P15 revealed a lasting migration deficit resulting from *GRM2* knowndown (**Supplementary Figure 1C**) indicating the pathogenic effects of disrupted mGluR2 functioning. Additionally, preliminary comparison of cell membrane expression levels of wildtype versus mutated mGluR2 indicated reduced cell membrane surface expression (**Supplementary Figure 2**).

The results of the radioactive displacement experiments indicate, with binding of [3H]LY341495 and displacement thereof using glutamate, that there is expression of mGluR2 on the cell membranes, although expression seems lower compared to wildtype *GRM2* receptors under the assumption of equal transfection efficiency (**Figure 3A and B**). Lower level of binding to mGluR2_R750H compared to reference mGluR2 suggests that the receptor expression is reduced and/or the orthosteric binding site is changed. Reduced receptor expression is more likely given that glutamate affinity seems unchanged. Glutamate can activate mGluR2_R750H though to a lower extent than the wildtype mGluR2 protein. This can solely be due to lower expression of mGluR2 and/or to a change of the receptor binding site or of downstream signalling. Lower expression seems more likely since glutamate potency seems unaltered. Also, there was reduced GTPγS signalling in the cells transfected with the mutant mGluR2 as compared to the wildtype mGluR2 (**Figure 4**). Western blot analyses confirm that mGluR2_R750H expression at the membrane is reduced (**Supplementary Figure 3**). mGluR2 positive allosteric modulators (PAMs) can still bind and potentiate a minimally effective glutamate concentration (**Supplementary Figure 4**).

Table 1 Clinical characteristics

Ped ID	II-1	II-2	III-1	III-2	III-3
DSM-IV Diagnosis	None	None	Bipolar I disorder	Bipolar I disorder	Schizophrenia, depressive disorder, panic disorder
Age at examination	72	74	46	47	44
Age of onset depressive episodes	Na	Na	36	43	41
Number of depressive episodes	Na	Na	1	1	1
Age of onset manic-psychotic episodes	Na	Na	36	33	Na
Number of manic-psychotic episodes	Na	Na	2	2	0
Age of onset psychotic episodes	Na	Na	Na	Na	41
Number of psychotic episodes	Na	Na	Na	Na	1
Medication	Na	Metformin	Lithium, quetiapine	Lithium	Haloperidol, citalopram
Other diagnoses	Na	Type II diabetes mellitus, High blood pressure	None	None	None
Educational level	Lower professional education	Primary education	Secondary education	Master's degree	Master's degree

Table 2 Exonic variants identified

Chr	1	1	2	3	3	3	3	5	5	5	13	15	16	17
Position	75038148	76288153	201436078	51750038	101571954	121575859	124492600	175956058	176918862	176931000	25029185	89172589	89590465	39657534
Ref	C	G	G	G	C	G	G	C	G	G	G	G	G	G
Alt	A	A	T	A	T	C	A	A	A	C	A	C	A	A
dbSNP 144	rs144055915	rs775625959	rs200938511	rs371451244	rs191580913	NA	rs757581313	NA	NA	rs754987743	NA	rs780362012	NA	rs569014208
Gene	*ERICH3*	*MYH4*	*SGOL2*	*GRM2*	*NFKBIZ*	*EAF2*	*ITGB5*	*RNF44*	*PDLIM7*	*DOK3*	*PARP4*	*AEN*	*SPG7*	*KRT13*
cDNA	c.3246G>T	c.1049G>A	c.1009G>T	c.2249G>A	c.584C>T	c.340G>C	c.1853C>T	c.1270G>T	c.344C>T	c.1475C>G	c.2728C>T	c.673G>C	c.428G>A	c.1351C>T
Protein	p.R1082S	p.R350Q	p.A337S	p.R750H	p.T195M	p.V114L	p.P618L	p.A424S	p.T115M	p.P492R	p.Q910*	p.G225R	p.R143H	p.R451C
ExAC missense tolerance score	-3.5	-1.07	-1.36	3.27	0.53	-0.41	0.61	0.17	0.34	-0.93	-0.68	-0.63	0.43	-0.65
ExAC LOF tolerance score	0	0	0	0.98	1	0	0	0.57	0.9	0	0	0	0	0
ExAC_ALL	0.0003	1.65E-05	0.0005	8.24E-06	8.26E-05	NA	8.25E-06	NA	NA	0.0002	NA	9.09E-05	NA	NA
ExAC_NFE	0.0005	3.00E-05	0.0008	1.50E-05	7.51E-05	NA	0	NA	NA	0.0003	NA	0.0002	NA	NA
SIFT score	0.72	0.19	0.46	0	0.01	0.39	0	0	0.15	0	0.29	0.01	0.54	0.08
SIFT prediction	T	T	T	D	D	T	D	D	T	D	T	D	T	T
Polyphen2 score	0.764	0.999	0.112	1	1	0.101	1	1	0.975	1	.	0.997	1	0.999
Polyphen2 prediction	P	D	B	D	D	B	D	D	D	D	.	D	D	D
LRT score	.	0	0.353	0	0	0.003	0	0	0.403	0.296	0.003	0.191	0	0.09
LRT prediction	.	D	N	D	D	N	D	D	N	U	N	N	D	N
MutationTaster score	1	1	1	1	0.914	0.982	1	1	1	1	1	1	1	0.998
MutationTaster prediction	N	D	N	N	D	N	D	D	N	N	A	N	D	N
MutationAssessor score	1.1	1.19	1.01	3.345	0.805	-0.345	2.155	1.435	1.01	0.975	.	2.62	1.5	0
MutationAssessor prediction	L	L	L	M	L	N	M	L	L	L	.	M	L	N
FATHMM score	2.64	-2.7	2.69	-2.8	0.36	.	-3.43	-0.28	2.36	1.31	.	2.01	-3.14	-1.68
FATHMM prediction	T	D	T	D	T	.	D	T	T	T	.	T	D	D
CADD phred	11.22	35	11.74	16.1	15.33	13.83	25.2	35	12.88	8.842	40	11.98	22.2	12.12

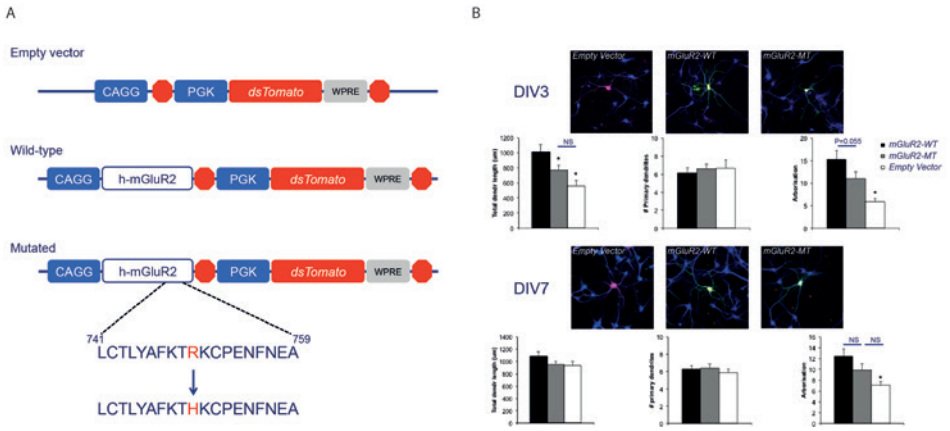


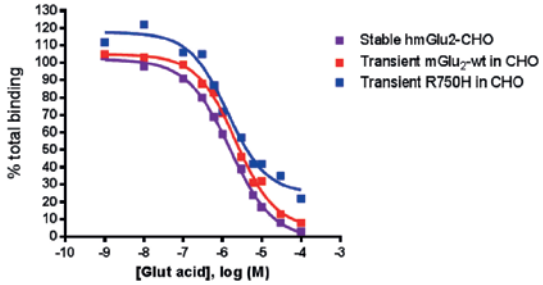
Figure 2 Transfection constructs and dissociated hippocampal neurons characteristics

A) Constructs used for the transfection of primary hippocampal neurons (empty vector top, wild-type middle, mutant bottom) **B top row)** Representative confocal images (20x objective, 0.5 zoom) of hippocampal neurons transfected on DIV3 either with control vector, WT mGluR2, or mGluR2p.R750H and fixated 5 days post-transfection. Transfected neurons are identified by the tdTOMATO (red). Neurons were stained with a specific mGluR2 antibody (green) and a microtubule (MAP2) antibody (blue). Merged pictures show clear colocalisation of the tdTOMATO with mGluR2 staining, indicating successful overexpression of the *GRM2* gene in these cells. **Second row, left)** Total dendritic length measured for each condition and normalized to the control vector. **middle)** Number of primary dendrites. **right)** Total number of branches (each parameter normalized to the control vector). Data are presented as mean ± SEM. **Third and fourth rows)** Same as for DIV 3, but now DIV 7. Statistical significance was assessed by one-way ANOVA followed by Tukey's post hoc test (* $p<0.05$).

These findings together indicate a loss-of-function effect of the *GRM2*_R750H substitution at the level of the protein.

Discussion

Through linkage analysis coupled with exome sequencing in a Dutch family with bipolar disorder, we identified a disease-segregating missense variant in *GRM2* which codes for the metabotropic glutamate receptor, type II (mGluR2). mGluR2 receptors are G-protein coupled receptors that use glutamate as neurotransmitter. mGluR2 has been found to be expressed on presynaptic terminals and postsynaptic spines, as well as on neuroglia. This highlights their modulating role with regard to excitatory signalling of glutamate, a pathway previously implicated in severe mental illness.[14]



	Stable	WT	R750H
Sigmoidal dose-response (variable slope)			
Best-fit values			
Bottom	-1.299	5.002	24.98
Top	102.1	104.8	117.5
LogEC50	-5.808	-5.660	-5.867
HillSlope	-0.7878	-0.8671	-0.8856
EC50	1.555e-006	2.187e-006	1.359e-006

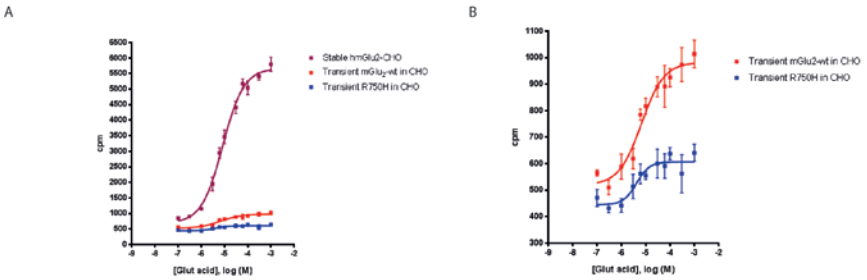
Figure 3 Autoradiography [^{3}H]LY341495 displacement by glutamate

CHO cells transiently transfected with wildtype or mutated mGluR2 and stably-transfected CHO cells with wildtype mGluR2. [^{3}H]LY341495 can be displaced by glutamate but is reduced in mGluR2_R750H transfected cells.

Previous research has implicated mGluR2 in the aetiology of psychosis by showing that mGluR2 interacts with serotonin receptor 2A (5-HTR2A) to form a heterodimer. The authors performed experiments showing a peculiar pattern of signalling of the mGluR2-5HTR2A dimer: in the physiological state there is more glutamatergic signalling than serotonergic signalling while this balance shifts upon administration of hallucinogenic compounds. Co-administration of mGluR2A agonists abolished this effect.[15]

The mGluR2 protein has been a major candidate protein and a very interesting target for drug development. Initial phase 2 clinical trials showed a reduction of positive and negative symptoms in patients with schizophrenia.[16] It might be very interesting to perform such a trial in patients with bipolar disorder with mGluR2 mutations with a demonstrated loss-of-function effect such as we presented here. Potentially, for this subpopulation of patients, there pharmacological intervention would be more beneficial than the standard pharmacotherapeutic regimen.

In conclusion, we described a missense variant in *GRM2* segregating with disease in a Dutch family with bipolar disorder and presented converging genetic and functional data involving the gene and its encoded protein in the aetiology of bipolar disorder through a loss-of-function mechanism. Screening of this gene is warranted in patients with familial bipolar disorder.



	Stable	WT	R750H
Sigmoidal dose-response (variable slope)			
Best-fit values			
Bottom	887.2	519.3	445.2
Top	5676	982.7	607.0
LogEC50	-5.079	-5.175	-5.379
HillSlope	0.9531	0.9364	1.618
EC50	8.344e-006	6.684e-006	4.181e-006

Figure 4 [^{35}S]GTPγS binding in response to glutamate

A) CHO cells transiently transfected with wildtype or mutated mGluR2 and stably-transfected CHO cells with wildtype mGluR2. [^{35}S]GTPγS binding is reduced in mGluR2_R750H transfected cells. **B)** Zoom in of **(A)** to indicate the CHO cells transiently transfected with wildtype or mGluR2_R750H only.

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Supplementary Table 1 Linkage regions under an autosomal dominant model

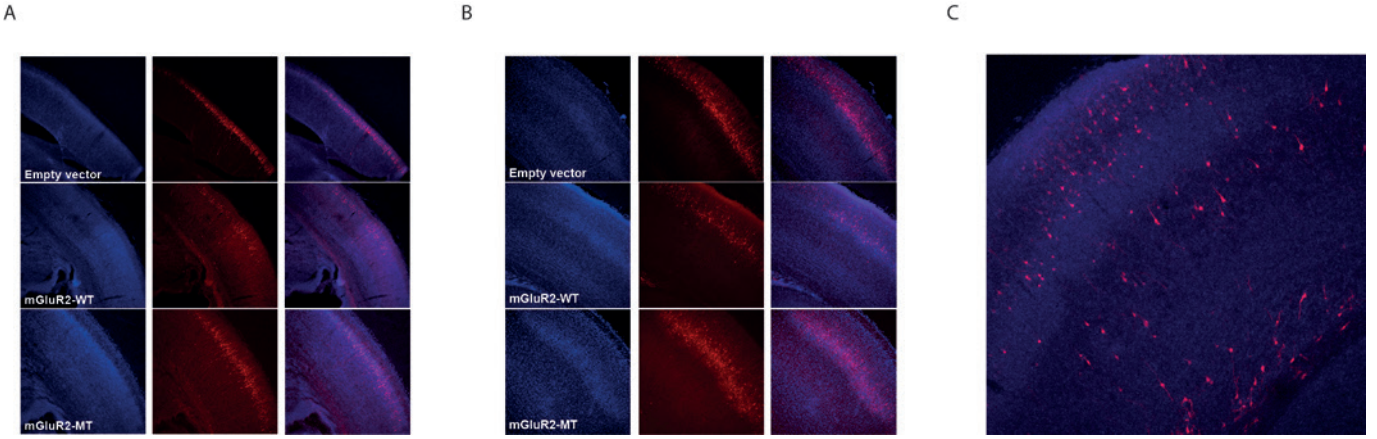
Chr	Start SNP	End SNP	Start (hg19)	Stop	Size in Mb
1	rs4301655	rs17350396	8313894	11900838	3.59
1	rs11209800	rs11166498	72066623	91895432	19.83
1	rs10797991	rs11117781	184848690	217334839	32.49
2	rs1477499	rs4674039	181470838	216794024	35.32
3	rs1161149	rs12635298	8487491	53033796	44.55
3	rs318738	rs1447698	64761884	134460368	69.70
3	rs2293151	rs406271	150285520	195776976	45.49
4	start	rs4516715	0	19847106	19.85
4	rs11945307	end	164196370	191154276	26.96
5	rs7727031	rs919739	147255920	167560809	20.30
5	rs10069570	end	172797393	180915260	8.12
6	start	rs7748189	0	18385889	18.39
6	rs16894454	rs6913076	64287871	99763266	35.48
6	rs11155587	rs9356458	149013861	166667434	17.65
7	start	rs2723437	0	13492885	13.49
7	rs4720271	rs847580	38267754	97670154	59.40
7	rs4731759	rs10952761	130839735	148179678	17.34
8	rs2439630	end	96245252	146364022	50.12
9	rs11137930	rs3001116	81497316	113404293	31.91
9	rs7028259	end	136061648	141213431	5.15
10	rs7089520	rs4745988	39097912	71155633	32.06
10	rs3892212	rs4917503	102062074	109323805	7.26
11	rs147570	rs885442	61597212	76920038	15.32
11	rs994781	rs595641	112510051	114017753	1.51

12	rs1344454	rs1379474	8995211	23331473	14.34
12	rs12372796	rs2372391	94517909	98968227	4.45
13	start	rs942364	0	28896097	28.90
14	rs4073416	rs7146787	66259394	75696922	9.44
14	rs10147069	end	95775877	107349540	11.57
15	start	12902711	0	27222824	27.22
15	rs2470893	rs7180119	75019449	94193200	19.17
16	rs12934904	rs7190492	23913393	53828752	29.92
16	rs4843344	rs164741	86114829	89692298	3.58
17	rs6502186	rs6502327	12190713	13946187	1.76
17	rs4794776	rs199494	36480609	44869063	8.39
18	rs9947735	rs17072617	45874561	61822210	15.95
19	start	rs918484	0	4301845	4.30
19	rs8112667	rs10469470	15133926	17187518	2.05
20	rs6118469	rs6079947	9055820	15710270	6.65
20	rs6103031	rs6095934	41381215	49049249	7.67
20	rs6099313	157091	55452732	56251561	0.80
21	start	rs374916	0	15550435	15.55
22	rs2285858	rs165626	19120959	20954760	1.83
				Total	844.80

Supplementary Table 2 *GRM2* primers

Variant	
GRM2_R750H_F	GGCACAGGCAAGGAGACAGC
GRM2_R750H_R	ATTCAGCCCTCCCACCTTCC
Open Reading Frame	
GRM2_Ex2_F	TCGCATCTCTCTTCTTGTCTGTCC
GRM2_Ex2_R	TGTGACCAGGGCACTTTCTTAGC
GRM2_Ex3(I)_F	TGTGGACACTTGACACCAAGACC
GRM2_Ex3(I)_R	GCCTGCCACCACACTCTCC
GRM2_Ex3(II)_F	TGGCCACCTCGGAGAAAGTG
GRM2_Ex3(II)_R	GCCTAGCATCCTTGCCCTTG
GRM2_Ex4(I)_F	TTAGCTCTGGCATTTGGGTTCC
GRM2_Ex4(I)_R	GGATGTACTCCTGGGGCAGTTC
GRM2_Ex4(II)_F	GAAGTCTGCTGCTGGCTCTGC
GRM2_Ex4(II)_R	TGTGAGGCAGGACTGATGAAGC
GRM2_Ex4(III)_F	TGCTACTCAGCCCTGCTCACC
GRM2_Ex4(III)_R	TCCCCTGAGTGGGCATTATCC
GRM2_EX5_F	CACCCTGGGAAAATGGTCTGG
GRM2_EX5_R	GGGAAGCAGGGCTGTGAGG
GRM2_Ex6_F	TCTCTGCCTGTTCCCCTCTCC
GRM2_Ex6_R	ACTTTGGGGGCAGACATAGGG

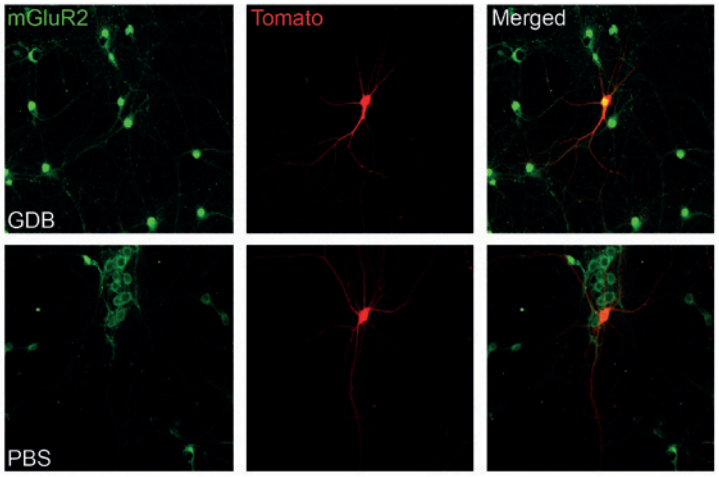
Supplementary Figures



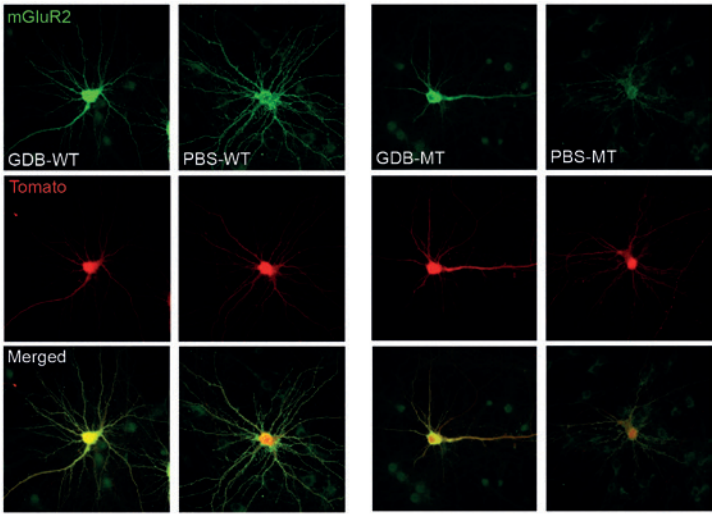
Supplementary Figure 1 In-utero electroporation performed at E14.5 and neuronal migration

A) Representative images of the developing somatosensory cortex obtained from coronal brain sections of P0 pups in utero electroporated at E14.5 with the following constructs: Empty vector, WT *GRM2,* or MT-*GRM2*p.R750H. tdTOMATO positive cells represent neurons successfully targeted. DAPI (blue) counterstaining is used to identify general cortical structure. **B)** Developing somatosensory cortex obtained from coronal brain sections of P7 pups in utero electroporated at E14.5 with the same constructs as **(A)**. **C)** Developing somatosensory cortex obtained from coronal brain sections of P0 pups in utero electroporated at E14.5 with shRNA against mGluR2; tdTOMATO positive cells represent neurons successfully targeted. DAPI (blue) counterstaining is used to identify general cortical structure.

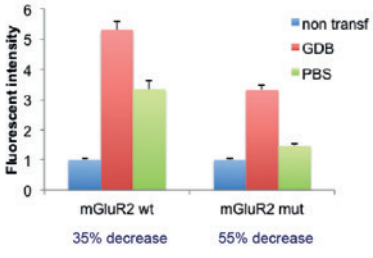
A


mGluR2
Tomato
Merged
GDB
PBS

B

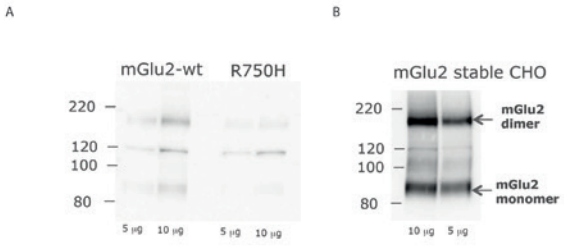

mGluR2
GDB-WT
PBS-WT
GDB-MT
PBS-MT
Tomato
Merged

C


Fluorescent intensity
6
5
4
3
2
1
0
non transf
GDB
PBS
mGluR2 wt
35% decrease
mGluR2 mut
55% decrease

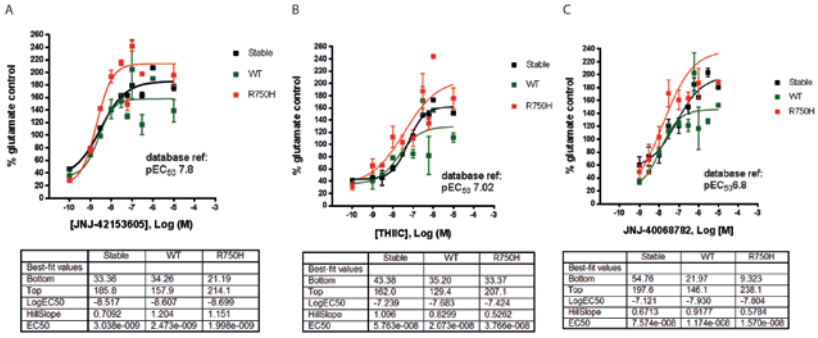
Supplementary Figure 2 Fraction of membrane insertion of mGluR2

A) MgluR2 antibody (first column) and Tomato staining (second column) staining using either Gelatin Dilution Buffer (GDB, with triton, top row) or phosphate buffered saline (PBS, bottom row) whereby the PBS shows the membrane-bound mGluR2 staining in empty vector transfection. **B)** MgluR2 antibody (first row) and Tomato (middle row) staining using either Gelatin Dilution Buffer (GDB, with triton, first column) or phosphate buffered saline (PBS, second column) whereby the PBS shows the membrane-bound mGluR2 staining. **C)** Quantification of total (GDB) and membrane-bound (PBS) mGluR2 fluorescent intensity (normalised for Tomato) indicating both a reduction in total mGluR2 expression as well as reduced membrane insertion of the GRM2_p.R750H construct.



Supplementary Figure 3 Western blot for mGluR2 enriched for membrane fraction

Western blot indicating reduced expression of mGluR2 monomer and dimer on CHO cell membrane transfected transiently with either wildtype or mutant mGluR2 **(A)** and stably transfected with wildtype mGluR2 **(B)**.



	Stable	WT	R750H
Best-fit values			
Bottom	33.36	34.26	21.19
Top	185.8	157.9	214.1
LogEC50	-8.517	-8.607	-8.699
HillSlope	0.7092	1.204	1.151
EC50	3.038e-009	2.473e-009	1.998e-009

	Stable	WT	R750H
Best-fit values			
Bottom	43.38	35.20	33.37
Top	162.0	129.4	207.1
LogEC50	-7.239	-7.883	-7.424
HillSlope	1.096	0.8299	0.5082
EC50	5.763e-008	2.073e-008	3.766e-008

	Stable	WT	R750H
Best-fit values			
Bottom	54.76	21.97	9.323
Top	197.8	146.1	238.1
LogEC50	-7.121	-7.930	-7.804
HillSlope	0.6713	0.9177	0.5784
EC50	7.574e-008	1.174e-008	1.570e-008

Supplementary Figure 4 Effect of mGluR2 positive allosteric modulators on [^{35}S]GTPγS binding

CHO cells transiently transfected with wildtype or mutated mGluR2 and stably-transfected CHO cells with wildtype mGluR2. [^{35}S]GTPγS binding is increased in increased in mGluR2_R750H transfected in response to 4 µM glutamate using the compounds JNJ-42153605 (**A**), THIIC (**B**), and JNJ-40068782 (**C**) which function as positive allosteric modulators.

Part II |

Neurodevelopmental disorders

4 |

Compound heterozygous variants in *SLC39A7* associated with non-syndromic familial autism spectrum disorder

Christian G. Bouwkamp[1,2], Geeske M. van Woerden[3], Anneke J.A. Kievit[2], Hella Hendriks-van der Heide[4], Simone Olgiati[2], Melissa A. Ploeg[3], Guido J. Breedveld[2], Marialuisa Quadri[2], Mark van der Kroeg[1], Wilfred van IJcken[5], Caroline Nava[6], Alexis Brice[6,7], Christel Depienne[6], Ype Elgersma[3], Femke M. de Vrij[1], Vincenzo Bonifati[2], Steven A. Kushner[1]

1 Department of Psychiatry, Erasmus MC, Rotterdam, The Netherlands
2 Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands
3 Department of Neuroscience, Erasmus MC, Rotterdam, The Netherlands
4 De Waerden, Alkmaar, The Netherlands
5 Centre for Biomics, Erasmus MC, Rotterdam, The Netherlands
6 University Pierre et Marie Curie, Paris, France
7 National Reference Centre for Neurogenetics, Federation of Neurology, Pitié-Salpêtrière University Hospital.

Manuscript in preparation

Abstract

Background: Autism spectrum disorders (ASD) comprise a heterogeneous group of childhood-onset psychiatric disorders affecting different domains of social interaction. ASDs are known to be highly heritable with a pluriform genetic aetiology. We studied a consanguineous Dutch Caucasian multiplex family with non-syndromic ASD.

Methods: We performed linkage analysis coupled with exome sequencing under an autosomal recessive model. The candidate variants were confirmed by Sanger sequencing and we attempted genetic validation in a cohort of 18 Dutch and 96 French ASD patients. Functional work consisted of overexpression experiments in dissociated mouse hippocampal neurons as well as in-vivo migration experiments in mouse hippocampus.

Results: We identified segregating, compound heterozygous variants in *SLC39A7* encoding the zinc transporter ZIP7. Existing functional evidence strongly implicates ZIP7 in early brain development, making this a plausible candidate gene for ASDs. Sequencing the open reading frame (ORF) of 228 chromosomes of patients with ASD did not identify additional rare coding variants. Preliminary functional evidence indicates a possible loss-of-function mechanism for both of the variants.

Conclusions: We propose *SLC39A7* as a candidate gene for non-syndromic ASD based on our genetic data and bot our own preliminary, and published functional work. Follow-up studies are required to definitively establish mutations in *SLC39A7* as a genetic determinant of ASD.

Introduction

Autism Spectrum Disorders (ASD) are a group of childhood-onset psychiatric disorders. They represent a variety of disorders at the clinical level, affecting the domains of social communication and interaction, with preference for sameness regarding behaviours, interests, and activities. With the recent introduction of the DSM-V, the distinction between the different autism spectrum disorders such as autistic disorder and Asperger's disorder has been abolished. Currently, all these disorders fall on the ASD spectrum.[1]

From twin-based statistical genetic studies, as well as single nucleotide polymorphism (SNP) array-based heritability studies, it is known ASDs have a high heritability rate of ~0.8. The heritability estimate for ASDs is similar to those of schizophrenia and bipolar disorder and they together comprise the group of psychiatric disorder with the strongest genetic aetiological component.[2] The recent meta-analysis of twin studies on human traits indicated a heritability estimate of 0.6 for all pervasive developmental disorders[3], while the SNP-based heritability estimate is ~0.8.[2] A significant fraction of the knowledge of the genetics of autism is derived from rare genetic syndromes which present with comorbid ASD, such as Fragile X and Angelman syndrome.[4] Most cases of autism however have an apparent sporadic presentation.

With the development of next generation sequencing (NGS) technology, it has become possible to determine the nucleotide sequence of the coding regions of the genome (the exome), or the entire genome. This can be done for a child with ASD and for both of his or her confirmed biological parents. Non-transmitted variants present in the child, but absent from the parents are termed *de novo* and, depending on the functional effect on the protein, might be causally related to the disorder with which the child is affected. When using this method of identifying *de novo* variants, this is under the assumption that the ASD is caused by a *de novo* mutation. Sporadic presentation of illness can be due to many factors, amongst others inherited factors that are autosomal recessive and/or have a reduced penetrance. Nevertheless, genes which are resulting more often from unbiased genetic studies could be good candidates for diagnostic screening and functional assessment. Recently, two interesting papers came out describing the state of the art in this field. Iossifov and colleagues performed exome sequencing in 2500 autism simplex families from the Simon's Foundation cohort and found that in combination with *de novo* copy number variants (CNVs), *de novo* mutations explain about 1/3 of the autism cases and this figure is slightly higher in females.[5] Earlier, Yuen and

colleagues, by using whole genome sequencing in quartets (parents and two affected offspring), discovered that when considering both *de novo* and inherited genetic variants, the two affected siblings carried largely different genetic risk alleles for developing ASD.[6] In addition, the different genetic risk allele combination also gave rise to slightly different clinical symptomatology.

In order to identify potentially novel genetic factors related to the development or the vulnerability to develop ASD, we initiated an ascertainment program for families with multiple members with ASD. In these families, we employed linkage analysis coupled with exome sequencing in accordance with recent literature guidelines.[7,8] Here, we describe a sibship of five boys born from consanguineous parents of whom four out of five have a diagnosis of ASD. Under an autosomal recessive linkage model, we identified compound heterozygous variants in *SLC39A7* that segregate with ASD. We propose this gene as a candidate gene for ASD.

Methods

Molecular diagnostic work-up and genetic counselling

As part of the clinical genetic diagnostic work-up, we performed a dysmorphological examination, SNP-array testing for genomic abberations related to ASD, metaphase karyotyping, Fragile-X testing by Southern blot, and routine metabolic screening in blood plasma and urine. The dysmorphological examination did not provide evidence for a syndromic presentation of ASD and all other laboratory screens were normal. We then pursued linkage analysis coupled with exome sequencing in order to identify novel genetic factors that might be causal for ASD in this family. Written informed consent was obtained from all participating subjects. This study was approved by the medical ethical committee of the Erasmus University Medical Centre (Rotterdam, The Netherlands).

Genetic analyses

Linkage and Copy Number Analysis was performed with Illumina HumanOmniExpress 700k SNP-arrays with DNA isolated from venous blood assuming an autosomal recessive affected-only model. Linkage analysis was conducted for the purpose of identifying chromosomal regions shared by all affected family members. Whole exome sequencing was performed at 86x average coverage in two affected siblings (IV-1 and IV-2). Exonic coding variants were only considered if they occurred within the defined regions of suggestive linkage, and showed co-

segregation with the disease in the family. Linkage was performed using Merlin and copy number variant (CNV) analysis was performed using NEXUS discovery edition, version 7 (BioDiscovery, El Segundo, CA).

Exome sequencing was performed using in-solution capturing (Agilent SureSelect V4 Human 50 Mb kit, Agilent Technologies) and paired-end sequencing on an Illumina Hi-Seq 2000 sequencer. Reads were aligned to the human reference genome version 19 using Burrows-Wheeler Aligner (BWA). SNPs and indels were called using the Genome Analysis Toolkit (GATK). Filtering of the variants was performed using Cartagenia software (Cartagenia Bench lab, Agilent Technologies).

Homozygous and double heterozygous variants were filtered based on the following criteria: a) present within the genomic regions shared among all affected family members, b) predicted to affect protein coding (missense, nonsense, frameshift, splice site), c) called in both affected individuals, d) absent from dbSNP129, and e) with a minor allele frequency (MAF) of < 0.05 in the more recent public databases (ExAC, Exome Variant Server 6500, 1000 Genomes, and Genome of the Netherlands). The variants were genotyped by Sanger sequencing of all participating family members to confirm their fidelity. Last, we performed Sanger sequencing of the open reading frame and exon-intron boundaries of *SLC39A7* in a cohort of 18 Dutch and 96 French patients with ASD (**Supplementary Table 1**).

Functional assays – neuronal transfection and in-utero electroporation

Constructs. The cDNA sequence from human *SLC39A7*-WT (NM_006979) and the variants found in the family (*SCL39A7*c.192_197 delTGGCCA and *SLC39A7*c.578T>A) were synthesized by GeneCust and cloned into a dual promoter expression vector (**Figure 3A**). The dual promoter expression vector was generated from the pCMV-*tdTomato* vector (Clontech), where the *CMV* promoter was replaced with a *CAGG* promoter followed by a multiple cloning site (MCS) and transcription terminator sequence. To assure expression of the tdTOMATO independent from the gene of interest, a PGK promoter was inserted in front of the *tdTomato* sequence. For all the *in vivo* and *in vitro* experiments, the vector without a gene inserted in the MCS was taken along as control (control vector).

Mice. For the neuronal cultures, FvB/NHsD females were crossed with FvB/NHsD males (both ordered at 8-10 weeks old from Envigo). For the *in utero* electroporation female FvB/NHsD (Envigo) were crossed with male C57Bl6/J (ordered at 8-10 weeks old from Charles River). All mice were kept group-housed in IVC cages (Sealsafe 1145T, Tecniplast)

with bedding material (Lignocel BK 8/15 from Rettenmayer) on a 12/12 h light/dark cycle in 21°C (±1°C), humidity at 40-70% and with food pellets (801727CRM(P) from Special Dietary Service) and water available *ad libitum*. All animal experiments were approved by the Local Animal Experimentation Ethical Committee, in accordance with Institutional Animal Care and Use Committee guidelines.

Primary hippocampal cultures. Primary hippocampal neuronal cultures were prepared from FvB/NHsD wild type mice according to the procedure described in [9]. Briefly, hippocampi were isolated from brains of E16.5 embryos and collected altogether in 10 ml of neurobasal medium (NB, Gibco) on ice. After two washings with NB, the samples were incubated in pre-warmed trypsin/EDTA solution (Invitrogen) at 37° for 20 minutes. After 2 times washing in pre-warmed NB, the cells were resuspended in 1.5 ml NB medium supplemented with 2% B27, 1% penicillin/streptomycin and 1% glutamax (Invitrogen), and dissociated using a 5 ml pipette. Following dissociation, neurons were plated in a small drop on poly-D-lysine (25 mg/ml, Sigma) coated 15 mm glass coverslips at a density of $1*10^6$ cells per coverslip in 12 well plates containing 1 ml of supplemented NB for each coverslip. The plates were stored at 37°/5% CO_2 until the day of the transfection.

Neuronal transfection and immunocytochemistry. Neurons were transfected at different days *in vitro* (DIV) with the following DNA constructs: control vector (1.8 ug per coverslip), *SLC39A7*-WT, *SLC39A7*p. p.G65_H66del, and *SLC39A7*p.L193H (all 2.5 ug per coverslip). Neurons were transfected at DIV3, DIV7 and DIV14. Transfection of the plasmids was done using Lipofectamine 2000 according to the manufacturer instructions (Invitrogen). For the neuronal morphology analysis, neurons were fixed 5 days post-transfection with 4% paraformaldehyde (PFA)/4% sucrose and incubated overnight at 4°C with primary antibodies in GDB buffer (0.2% BSA, 0.8 M NaCl, 0.5% Triton X-100, 30mM phosphate buffer, pH7.4). The following primary antibodies were used: guinea-pig anti MAP2 (1:500, Synaptic System) to stain for dendrites and rabbit anti ZIP7 (HPA053999 Atlas Antibodies). Secondary antibodies used were donkey anti-rabbit-Alexa488-, and donkey anti-guinea-pig-Alexa647-conjugated antibodies (all 1:200, Jackson ImmunoResearch). Slides were mounted using mowiol-DABCO mounting medium. Confocal images were acquired using a LSM700 confocal microscope (Zeiss).

For the analysis of the neuronal transfections, at least ten confocal images (20X objective, 0.5 zoom, 1024x1024 pixels) of different transfected neurons (identified by the red staining) were taken from each coverslip for each experiment with at least two independent replications. For

the analysis of the neuronal morphology, the NeuronJ plugin for ImageJ software was used to trace the dendrites with their branches. Total dendrite length, number of Primary dendrites and number of Branches were analysed. All the values were normalized against the mean value for each parameter of the control (control vector).

In-utero electroporation. The procedure was performed in pregnant FvB/NHsD mice at E14.5 of gestation to target mainly the progenitor cells giving rise to pyramidal cells of the layer 2/3.[10,11] The DNA construct (1.5-3 ug/ul) was diluted in fast green (0.05%) and injected in the lateral ventricle of the embryos while still *in uterus*, using a glass pipette controlled by a Picospritzer® III device. To ensure the proper electroporation of the injected DNA constructs (1-2 ul) into the progenitor cells, five electrical square pulses of 45V with a duration of 50 ms per pulse and 150 ms inter-pulse interval were delivered using tweezer-type electrodes connected to a pulse generator (ECM 830, BTX Harvard Appartus). The electrodes were placed in such a way that the positive pole was targeting the developing somatosensory cortex. The following plasmids were injected: control vector, *SLC39A7*_WT, *SLC39A7*_p.G65_H66del, and *SLC39A7*_p.L193H. After birth, pups were sacrificed at P0 or P7 for histochemical processing (to look at neuronal migration).

Immunohistochemistry. Mice were deeply anesthetized with an overdose of Nembutal and transcardially perfused with 4% paraformaldehyde (PFA). Brains were extracted and post-fixed in 4% PFA. Brains were then embedded in gelatin and cryoprotected in 30% sucrose in 0.1M PB, frozen on dry ice, and sectioned using a freezing microtome (40/50 μm thick). Free-floating coronal sections were washed in 0.1M PB and a few selected sections were counterstained with 4′,6-diamidino-2-phenylindole solution (DAPI, 1:10000, Invitrogen) before being mounted with mowiol on glass. Overview images of the coronal sections were acquired by tile scan imaging using a LSM700 confocal microscope (Zeiss) with a 10X objective. Zoom in images of the targeted area were taken using a 20X objective.

Statistical analysis. Statistical difference between the conditions for the in vitro experiments was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons.

Results

Family Ascertainment

We ascertained a Dutch white Caucasian family of which >4 generations were born in The Netherlands. The siblings were aged 12-18 at the time of ascertainment and four of the five

had a DSM-IV diagnosis of ASD. The oldest boy who was 18 at the time of ascertainment had a DSM-IV diagnosis of Autistic disorder diagnosed at the age 7, with an IQ of 78. The second sibling has a diagnosis of pervasive developmental disorder, not otherwise specified (PDD-NOS) with oppositional defiant disorder (ODD) diagnosed at the age of 8-9. His IQ is below average (Total IQ, TIQ 82) and he had lived in a home for ~3 years in order to manage his behavioural symptoms. The third sibling had a diagnosis of PDD-NOS with a TIQ of 96. He panicked if there was not sufficient daily structure provided. The last affected sibling had a diagnosis of PDD-NOS with attention deficit hyperactivity disorder (ADHD), as well as anxiety and depressive symptoms. He took methylphenidate and fluoxetine. His TIQ was IQ 102 (**Table 1**).

Copy number variant analysis and parametric linkage analysis

Analysis with the NEXUS software package did not reveal any copy number variants segregating with disease. Parametric linkage analysis under an autosomal recessive model showed ~49 Mb of shared genomic regions on the chromosomes 6, 11, 18, and 21 (**Supplementary Table 2**). These shared regions were followed-up with exome sequencing for variant discovery.

Exome sequencing

Analysis of the exome sequencing data revealed no homozygous variants, but did reveal compound heterozygous variants in three genes: *HLA-A* (c.142G>T p.A48S & c.497T>C p.I166T), *SLC39A7* (c.192_197delTGGCCA p.G65_H66del & c.578T>A p.L193H), and *AHNAK* (c.6718C>A p.H2240N & c.3463G>A p.V1155I) (**Table2**). We did not consider the variants in *HLA-A* and *AHNAK* plausible candidates since these are genes known to be highly polymorphic. However, we cannot exclude they are associated with the phenotype studied here. We identified compound heterozygous variants in the gene *SLC39A7*: c.192_197 delTGGCCA, p.G65_H66del and c.578T>A, p.L193H, whereby the deletion was inherited from the paternal grandfather and the SNV from the maternal grandmother (**Table 2, Figure 1, Figure 2A**). The unaffected sibling (IV-4) was carrier of the p.G65_H66 deletion only, but not of the p.L193H variant, a genotype shared by their father and paternal grandfather. The consequence of the 6 base-pair deletion on the paternal allele is an in-frame deletion of a glycine and a histidine residue at positions 65 and 66 respectively.

Table 1 Clinical characteristics

Individual	IV-1	IV-2	IV-3	IV-4
DSM-IV diagnosis	ASD (autistic disorder)	ASD (PDD-NOS), ODD	ASD (PDD-NOS)	ASD (PDD-NOS), ADHD
Age at recruitment	18	16	13	11
Medication	None	Fluoxetine	None	Pipamperone, atomoxetine, fluoxetine
IQ	TIQ 78	TIQ 82, VIQ 84, PIQ 82	TIQ 96, VIQ94, PIQ99	TIQ 102
Additional information	Special education	Trials with pipamperone, haloperidol, risperidone, methylphenidate, fluoxetine. Only risperidone alleviated symptoms @8y;4m →TIQ 82, VIQ 84, PIQ 82 @6y;5m→TIQ 81, VIQ 85, PIQ 81	@ 6y;5m→ TIQ 96, VIQ 94, PIQ 99	@ 4y;7m→ echolalia and psychomotor retardation

TIQ Total IQ, VIQ verbal IQ, PIQ performance IQ

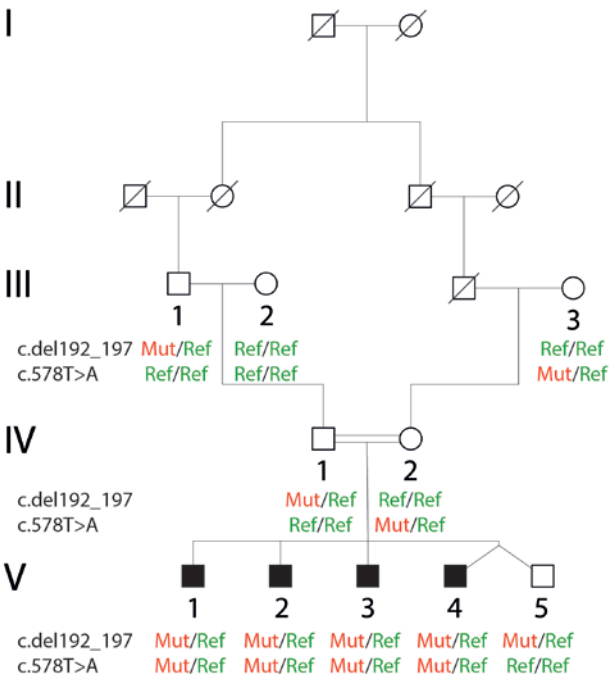


Figure 1: Family pedigree and *SLC39A7* variants segregation

Shaded symbols indicate family members with autism spectrum disorder (ASD) Subjects of whom DNA was available are numbered. Males are represented with squares and females are represented with circles.

This potentially leads to the production of an unstable protein. On the maternal allele, the patients have a missense mutation at position 193 that is predicted deleterious by Polyphen2 prediction software. In the largest public database to date, the Broad Exac database, the p.G65_H66del has a minor allele frequency (MAF) in non-Finnish Europeans of 3.0 $X10^{-4}$ (20/66706 alleles) while the MAF of the p.L193H mutation in Non-Finnish Europeans was 3.2 X 10^{-5} (2/63354 alleles). The amino acid sequence at the site of the variants is strongly conserved especially in mammals (**Figure 2B**), allowing for the study of these specific variants in these organisms.

Sequencing of the open reading frame of SLC39A7 in ASD cases

We performed Sanger sequencing of the open reading frame and exon-intron boundaries of *SLC39A7* in 18 Dutch patients and 96 French patients with ASD enriched for familial cases in an effort to find other rare coding variants in this gene associated with ASD.

Table 2 Identified exonic variants (GRCh37/hg19)

Chr	Start	Stop	Ref	Alt	dbSNP ID	Gene	cDNA change	Protein change
6	29910602	29910602	G	T	rs41552219	HLA-A	c.142G>T	p.A48S
6	29911198	29911198	T	C		HLA-A	c.497T>C	p.I166T
6	33169205	33169210	CCATGG	.		SLC39A7	c.192_197delTGGCCA	p.G65_H66del
6	33169688	33169688	T	A	rs373509303	SLC39A7	c.578T>A	p.L193H
11	62295171	62295171	G	T		AHNAK	c.6718C>A	p.H2240N
11	62298426	62298426	C	T		AHNAK	c.3463G>A	p.V1155I

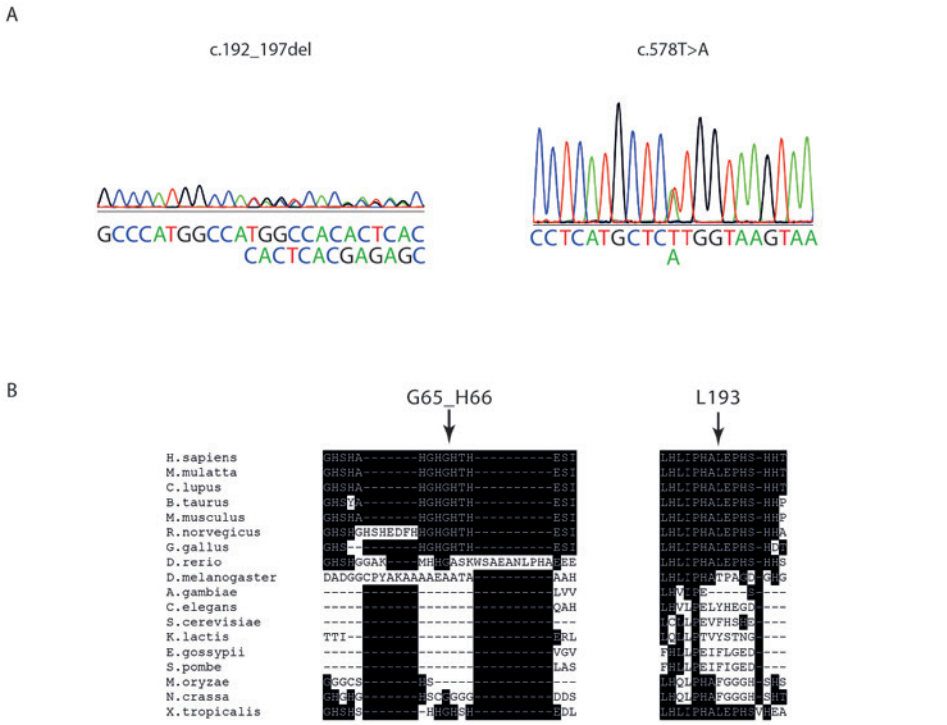


Figure 2: Electropherograms of *SLC39A7* variants and evolutionary conservation of ZIP7 protein

A) Electropherograms indicating the compound heterozygous variants (paternal c.192_194 deletion left panel; maternal c.578T>A single nucleotide variant right panel) **B)** Amino acid conservation within the ZIP7 protein homologs across species.

This work revealed several variants in the gene, but none affected the coding sequence and had a MAF <0.001 (as were the criteria in the initial discovery family).

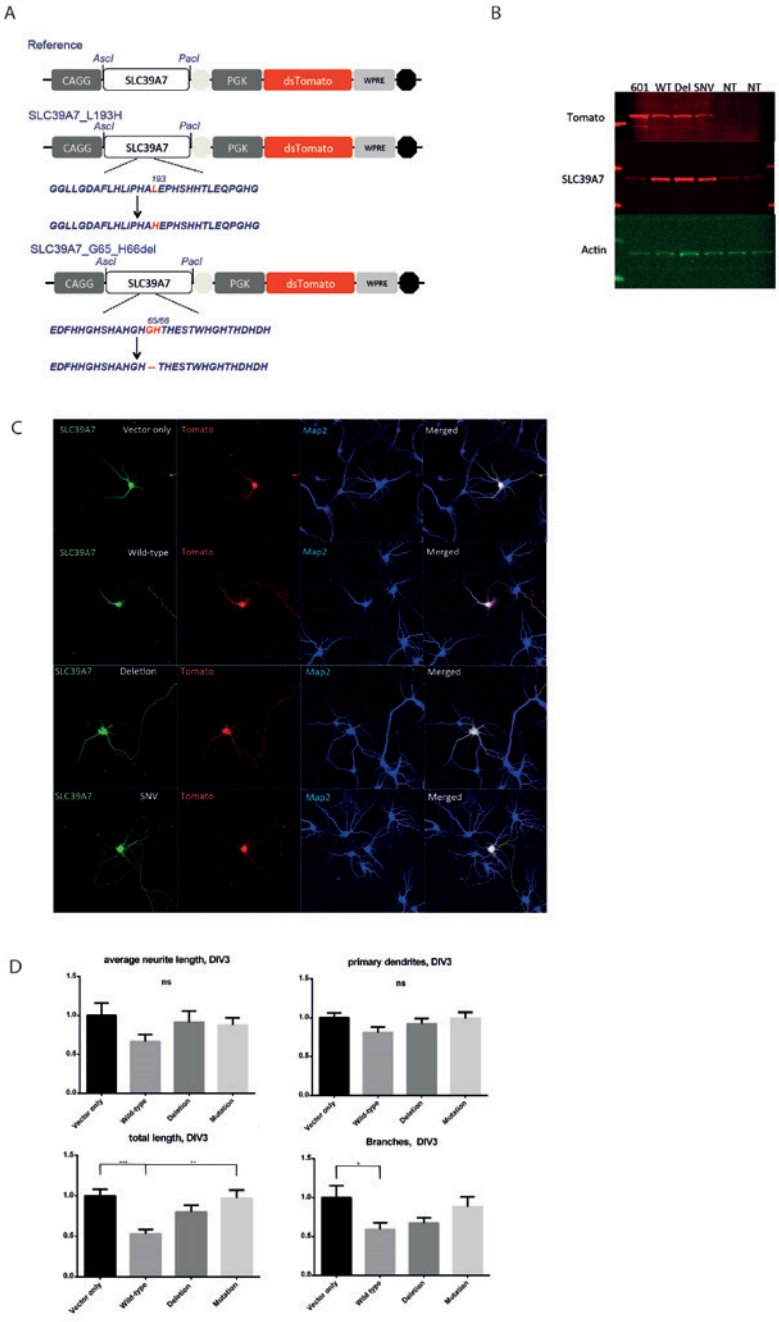
Functional experiments

Cells were transfected with equal efficiency for the *SLC39A7* wildtype and mutant constructs (**Figure 3B**). The preliminary results of the neuronal transfection experiments at three days in-vitro (DIV3) indicate that, in neurons transfected with the wildtype *SLC39A7* construct, the total dendrite length was decreased compared to the neurons transfected with the empty vector (one-way ANOVA, $p = 0.002$, $F(3, 66) = 7.568$; empty vector vs WT *SLC39A7*: $p = 0.0004$). In neurons transfected with the *SLC39A7*_p.G65_H66del variant-construct however, this effect was blunted (empty vector vs *SLC39A7*_p.G65_H66del: $p = 0.29$) (**Figure 3C and D**). This effect was even stronger in the neurons transfected with the *SLC39A7*_p.L193H

variant construct (empty vector vs: *SLC39A7*_p.L193H p = 0.996) (**Figure 3C and D**). After 7 in-vitro (DIV7), the differences in total dendrite length were ameliorated (**Supplementary Figure 1**). In addition, an increased primary dendrite length was observed in the neurons transfected with the *SLC39A7*_p.G65_H66del variant-construct compared to transfection with *SLC39A7* wildtype (one-way ANOVA, p = 0.0469, F(3, 86) = 2.762; WT SLC39A7 vs *SLC39A7*_p.G65_H66del: p = 0.036). For the number of branches, it was observed that there was an increased number in neurons transfected with the *SLC39A7*_p.G65_H66del mutant construct compared to neurons transfected with *SLC39A7* wildtype or *SLC39A7*_p.L193H construct (one-way ANOVA, p = 0.0093, F(3, 86) = 4.073; empty vector vs WT *GRM2*: WT SLC39A7 vs *SLC39A7*_p.G65_H66del p = 0.0095; *SLC39A7*_p.G65_H66del vs *SLC39A7*_p.L193H p=0.0366). Similarly, the preliminary in-utero migration assay data demonstrated there was significant disruption of neuronal migration in the mice transfected with the either *SLC39A7*_G65_H66del or *SLC39A7*_L193H variant-construct (**Supplementary Figure 2**).

Discussion

We performed linkage analysis coupled with exome sequencing in a consanguineous family where 4 siblings were affected with ASD. We identified fully segregating compound heterozygous variants in *SLC39A7*, which potentially have a deleterious effect on the protein. *SLC39A7* encodes the protein ZIP7, a zinc transporter expressed on the Golgi apparatus, which transports zinc from within the Golgi apparatus to the cytoplasm. The protein expression is regulated by zinc levels and is expressed ubiquitously in human tissues.[12] Our preliminary overexpression experiments show a reduced effect on neuronal morphology obtained by transfection with reference sequence by both the *SLC39A7*_p.G65_H66del and *SLC39A7*_p.L193H constructs compatible with a loss-of-function mechanism whereby the effect of the *SLC39A7*_p.L193H variant was stronger than the *SLC39A7*_p.G65_H66del variant. Together, the preliminary functional work indicated the identified variants in SLC39A7 likely have a pathogenic effect on the protein functioning. Additionally in adult zebrafish, it has been shown that ZIP7 is expressed most abundantly in the brain and the eye. In addition, the highest expression of the mRNA was in development up till 12 hours post-fertilization. The morpholino knock-down resulted in abnormal development of the oro-facial region and the spinal chord. Furthermore, the zinc levels in brain and eyes were reduced.


A
Reference
AscI
PacI
CAGG
SLC39A7
PGK
dsTomato
WPRE
SLC39A7_L193H
193
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GGLLGDAFLHLIPHAHEPHSHHTLEQPGHG
SLC39A7_G65_H66del
65/66
EDFHHGHSHAHGHGHTHESTWHGHTHDHDH
EDFHHGHSHAHGH -- THESTWHGHTHDHDH
B
601 WT Del SNV NT NT
Tomato
SLC39A7
Actin
C
SLC39A7
Vector only
Tomato
Map2
Merged
Wild-type
Deletion
SNV
D
average neurite length, DIV3
primary dendrites, DIV3
total length, DIV3
Branches, DIV3
ns
Vector only
Wild-type
Deletion
Mutation

Figure 3: Functional experiments 3 days in-vitro

A) Constructs used for the transfection of primary hippocampal neurons (wildtype *SLC39A7*, *SLC39A7*_L193H variant middle, *SLC39A7*_G65_H66del variant bottom) **B)** Western blot showing transfection efficiency in primary hippocampal cells with control vector, reference *SLC39A7*, *SLC39A7*_p.G65_H66del variant or *SLC39A7*_p.L193H variant and two non-transfected cell lysates **C)** Representative confocal images (20x objective, 0.5 zoom) of hippocampal neurons transfected on DIV3 either with control vector (**top row**), wildtype *SLC39A7* (**second row**), *SLC39A7*_G65_H66del (**third row**) or *SLC39A7*_p.L193H (**bottom row**) and fixated 5 days post-transfection. Transfected neurons are identified by the tdTOMATO (red). Neurons were stained with a specific ZIP7 antibody (green) and a microtubule (MAP2) antibody (blue). Merged pictures show clear colocalisation of the tdTOMATO with ZIP7 staining, indicating successful overexpression of the *SLC39A7* gene in these cells. **D)** Average neurite length, total dendrits, total dendrite length, and total number of branches (all measurements counted for each condition and normalized to the control vector) Data are presented as mean ± SEM. Statistical significance was assessed by one-way ANOVA followed by Tukey's post hoc test (* $p<0.05$).

These developmental abnormalities could be rescued by supplementation of zinc to the swimming water. mRNA levels of other members of the ZIP protein family (ZIP3 and ZIP6) as well as members of the ZnT family (ZnT2, ZnT5, and ZnT6) which are all involved in zinc homeostasis were upregulated in response to ZIP7 knockdown.[13] Interestingly, a *de novo* variant in *SLC30A5*, which codes for ZnT5, has been described in a family with ASD[14], which leads to the hypothesis that disruptions in the zinc metabolism pathway are potentially associated with a vulnerability of developing ASD. Based on our genetic and preliminary functional work as well as the existing literature on protein function, we propose *SLC39A7* as a candidate gene for non-syndromic ASD. We did not identify other rare variants which affected the coding sequence in a cohort of 18 Dutch and 96 French patients with ASD which suggests mutations in this gene are not a common cause of ASD. Further studies are required to definitively establish *SLC39A7* as a causative gene for ASD.

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Supplementary Tables

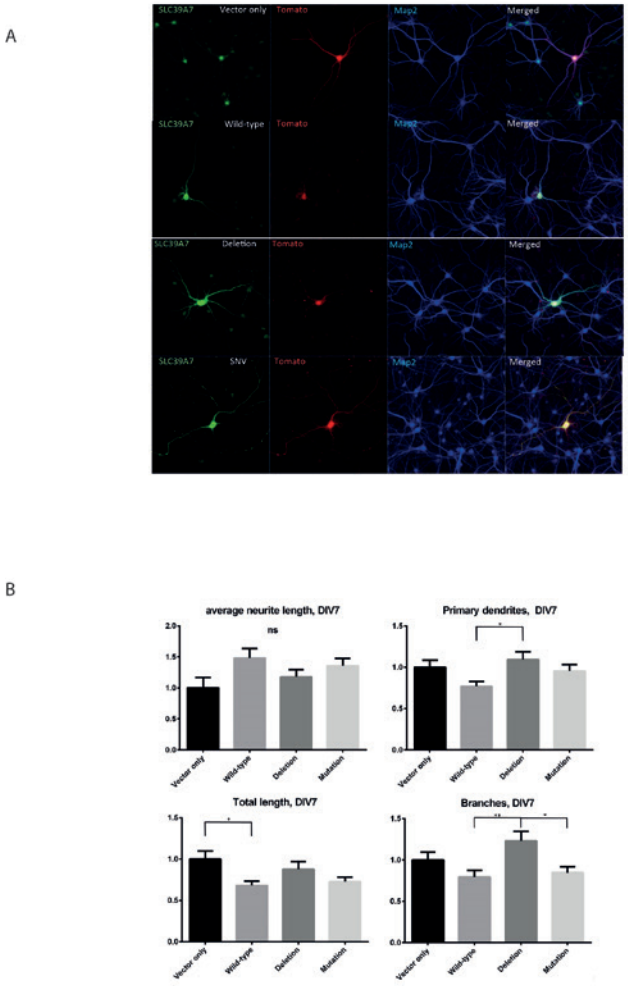
Supplementary Table 1 Primers *SCL39A7* open reading frame

Exon	Forward primer	Reverse primer
1	TACATCCGGGAGGTGGTTCG	CCCCAGTGCCTGGTGAGG
2 & 3	GAGCCTCTACCACAGAGGACATGG	CCAGACTCCTCCTCCTCAAGTGC
4	CAGATGGGGATGGGAGTTGG	GTCTCACTGGCCCATCTTTGG
5	GGAGAAATTTGTGAGACATGTGAAAGG	TCAGAGACCACTTTACACCCAGTCC
6	CCCAAAGATGGGCCAGTGAG	CCCTCATTTCTAGAGGACTTGTCAATG
7	TGATGTTTAGTTTCCAAATCCATGTCC	GGTCCCTCTGGCCTCTCATTC

Supplementary Table 2 Linkage regions autosomal recessive model (hg19)

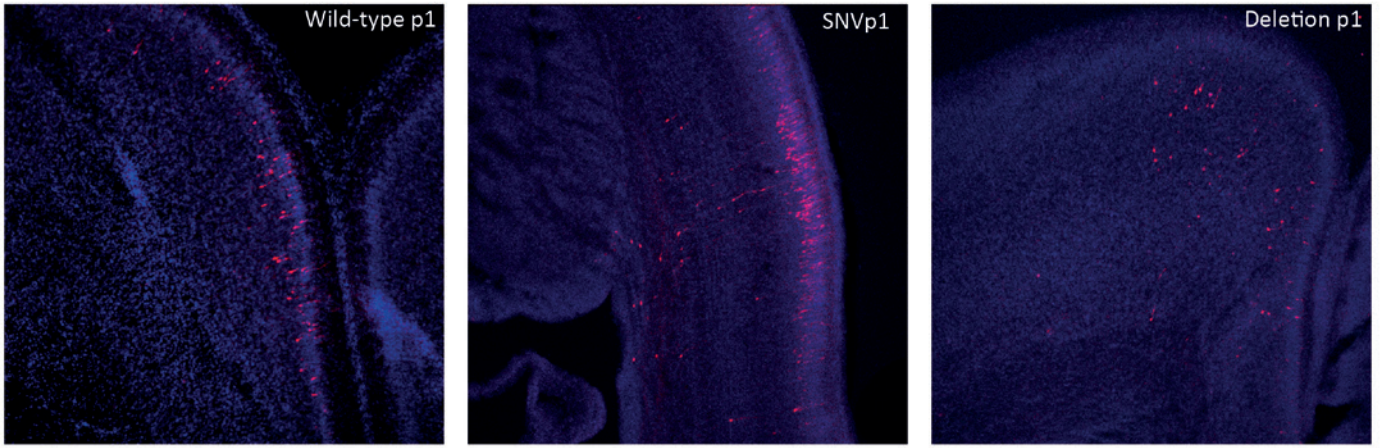
Chr	Left SNP	Right SNP	Left physical boundary	Right physical boundary	LOD score	Size in Mb	Size in cM
6	rs4712585	rs236410	21,108,114	36,755,402	1.81	15.65	12.83
11	rs4939451	rs4506683	60,537,069	70,906,073	1.81	10.37	12.02
18	rs17066203	rs12956791	57,357,320	64,568,005	1.81	7.21	10.75
21	start	rs437521	0	15,591,689	1.81	15.59	4.23
				Total		48.82	39.83

Supplementary Figures



Supplementary Figure 1: Functional experiments 7 days in-vitro

A) Representative confocal images (20x objective, 0.5 zoom) of hippocampal neurons transfected on DIV3 either with control vector (**top row**), wildtype *SLC39A7* (**second row**), *SLC39A7*_G65_H66del (**third row**) or *SLC39A7*_p.L193H (**bottom row**) and fixated 5 days post-transfection. Transfected neurons are identified by the tdTOMATO (red). Neurons were stained with a specific ZIP7 antibody (green) and a microtubule (MAP2) antibody (blue). Merged pictures show clear colocalisation of the tdTOMATO with ZIP7 staining, indicating successful overexpression of the *SLC39A7* gene in these cells. **B)** Average neurite length, number of primary dendrites, total dendritic length, total number of branches (each parameter counted for each condition and normalized to the control vector). Data are presented as mean ± SEM. Statistical significance was assessed by one-way ANOVA followed by Tukey's post hoc test (* $p<0.05$).



Supplementary Figure 2: In-utero electroporation

Representative images of the developing somatosensory cortex obtained from coronal brain sections of P1 pups in utero electroporated at E14.5 with either wildtype *SLC39A7* (left), *SLC39A7*_p.L193H (middle), or *SLC39A7*_G65_H66del (right). tdTOMATO positive cells represent neurons successfully targeted. DAPI (blue) counterstaining is used to identify general cortical structure.

5 |

ACO2 homozygous missense mutation associated with complicated hereditary spastic paraplegia

Christian G. Bouwkamp[1,2#], Zaid Afawi[3#], Aviva Fattal-Valevski[3,4#], Marialuisa Quadri[2], Guido J. Breedveld[2], Hanna Mandel[5], Muhammad Abu Tailakh[6], Wilfred F.J. van IJcken[7], Meike W. Vernooij[8,9], Vincenzo Bonifati[2*], Steven A. Kushner[1*]

1 Department of Psychiatry, Erasmus MC, Rotterdam, The Netherlands
2 Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands
3 Sackler School of Medicine, Tel-Aviv University, Ramat-Aviv, Israel
4 Pediatric Neurology Unit, Dana Children's Hospital, Tel-Aviv Medical Center, Tel-Aviv, Israel
5 Metabolic Disease Unit, Meyer Children's Hospital, Rambam Health Care Campus and Technion Faculty of Medicine, Haifa, Israel
6 Clinical Research Center, Nursing Research Unit, Soroka University Medical Center and Faculty of Health Science, Ben Gurion University of the Negev, Be'er Sheva, Israel
7 Center for Biomics, Erasmus MC, Rotterdam, The Netherlands
8 Department of Epidemiology, Erasmus MC, Rotterdam, The Netherlands
9 Department of Radiology, Erasmus MC, Rotterdam, The Netherlands
#,* These authors contributed equally

Manuscript submitted

ABSTRACT

Background: Hereditary spastic paraplegias (HSP) are a clinically and genetically heterogeneous group of neurodegenerative disorders. The identification of the disease-causing genes has been delineating a growing list of HSP forms, providing crucial insights into the underlying molecular mechanisms. However, in a significant proportion of cases, the causative gene remains unknown.

Methods: Clinical and genetic analysis, including genome-wide linkage coupled to whole exome sequencing, in a consanguineous family with complicated HSP.

Results: A homozygous novel missense mutation was identified in the *ACO2* gene (c.1240 T>G p.Phe414Val) that segregated with the disease in the family investigated. *ACO2* encodes the mitochondrial aconitase, an essential enzyme in the Krebs cycle. *ACO2* mutations have been previously described in rare patients with autosomal recessive developmental delay and cerebellar ataxia, with or without retinal degeneration. So far, *ACO2* mutations have not been associated with HSP.

Conclusions: Our findings profoundly expand the associated phenotype, and nominate *ACO2* as a novel disease-causing gene for autosomal recessive, complicated HSP. Our findings provide also further support for a central role of mitochondrial defects in the pathogenesis of HSP.

Introduction

Hereditary spastic paraplegias (HSP) are a clinically and genetically heterogeneous group of disorders characterized by neuronal degeneration of the corticospinal tracts, typically resulting in progressive weakness in the lower extremities and muscle spasms [1]·[2]. Gait difficulties are the most common presenting symptom with a mean age of onset of eight years old [3]. A recent meta-analysis estimated the overall prevalence of HSP to be approximately 3 per 100,000 people [4]. HSP can present as uncomplicated forms, limited to pyramidal tract (plus urinary) dysfunctions, or in complicated forms involving additional neurological or neuropsychiatric signs and symptoms [5]. Complicated HSPs are more often associated with either an autosomal recessive or X-linked pattern of inheritance [6].

To date, more than 80 chromosomal loci have been linked to HSP forms [6–8]. The majority of autosomal dominant forms is explained by mutations in *SPAST* (SPG4), *ATL1* (SPG3A) and *REEP1* (SPG31), while the majority of autosomal recessive HSP is explained by mutations in KIAA1840 (SPG11), *CYP7B1* (SPG5) and *SPG7* (SPG7). Nevertheless, in a substantial percentage of patients (38% in autosomal dominant HSP and 53% in autosomal recessive HSP) no causative mutations are identified in any of the known genes [7]. Here, we report the identification, by genome-wide unbiased approach, of a homozygous mutation in the *ACO2* gene in a consanguineous family with HSP, intellectual disability and microcephaly. To the best of our knowledge, recessive mutations in this gene were previously associated only with cerebellar ataxia with or without retinal degeneration in a very small number of patients. Our data expand markedly the associated phenotype and nominate *ACO2* as a gene causing a novel form of autosomal recessive complicated HSP.

Methods

Family Ascertainment

We ascertained a consanguineous Israeli family of Arab-Bedouin descent from Galilee, Northern Israel (**Figure 1A**) with two siblings affected with complicated spastic paraplegia. There were no affected relatives in the previous generations, in keeping with an autosomal recessive pattern of disease inheritance. This research was performed in accordance to the declaration of Helsinki and was approved by the institutional review board of the Sourasky University Medical Center, Tel-Aviv University and the Israeli ministry of Health. Written informed consent was obtained from the adult participants, with assent from the minors and written informed consent provided by their parents.

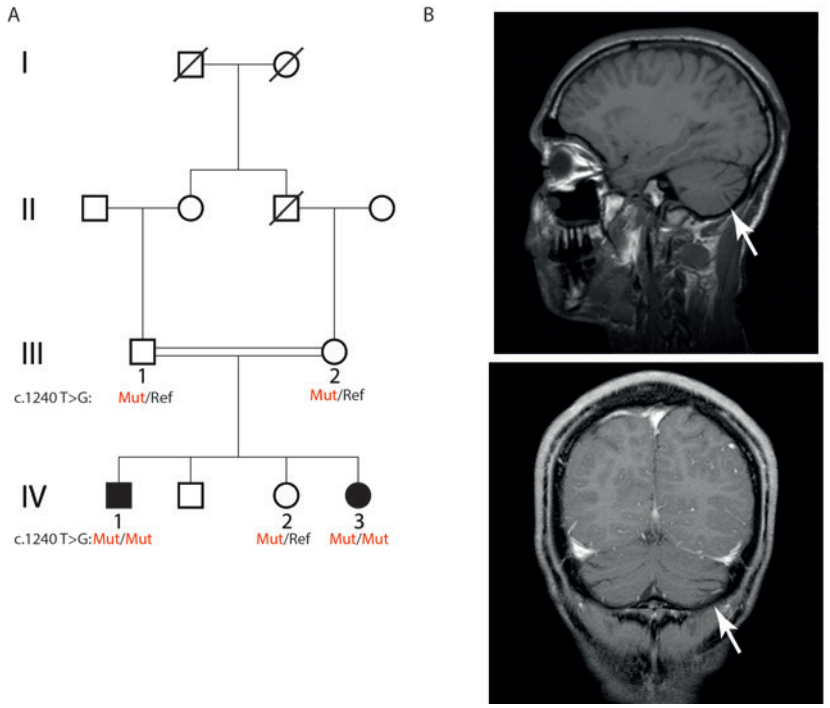


Fig 1: Family Pedigree and MRI imaging

A) Family pedigree. Shaded symbols indicate family members with complicated hereditary spastic paraplegia (HSP). Subjects of whom DNA was available are numbered. Males are represented with squares and females with circles. **B)** Sagittal T1-weighed MRI image (upper panel) and coronal T1-weighed MRI image (bottom panel) demonstrating mild cerebellar atrophy (arrows) in the proband (Ped ID IV-1).

Genetic analyses

Genomic DNA was isolated from venous blood using standard protocols. Genome-wide search for copy number abnormalities was performed using Illumina HumanOmniExpress-24 Beadchip 700k SNP-arrays and NEXUS discovery edition, version 7 (BioDiscovery, El Segundo, CA). Furthermore, the same SNP-arrays data were used to perform a genome-wide linkage scan by the program Merlin under the assumption of autosomal recessive disease inheritance and full penetrance (penetrance of known HSP genes is estimated at 0.9 [9]).

Whole exome sequencing in the two affected siblings was performed at ~100x average coverage, at the Center for Biomics of the Erasmus MC. The exome protocols included in-solution capturing (Agilent SureSelect V4 Human 50 Mb kit, Agilent Technologies) and paired-end sequencing on an Illumina Hi-Seq 2000 sequencer. Reads were aligned to the human reference genome version 19 using Burrows-Wheeler Aligner (BWA). SNPs and indels

were called using the Genome Analysis Toolkit (GATK). Filtering of the variants was performed using Cartagenia software (Cartagenia Bench lab, Agilent Technologies).

Variants were filtered based on the following criteria: a) located within the genomic loci supported by linkage analysis under an autosomal recessive model of disease inheritance (LOD score >0); b) predicted to affect protein coding (missense, nonsense, frameshift, splice site); c) called in both affected individuals; d) absent from dbSNP129; e) having a minor allele frequency (MAF) <0.001 in public databases (Exome Variant Server 6500 [EVS6500], 1000 Genomes, and Exome aggregation consortium [ExAC]). The resulting candidate variant was confirmed using direct (Sanger) sequencing (PCR primers: forward-TGCTCACTGTCTCCTCCTGACC and reverse-GGACAATGCCACCCAGATCC) in all participating family members from whom DNA was available.

Results

Clinical studies

The **proband** (**IV-1**) is a 28-year-old man, diagnosed with complicated spastic paraplegia, severe intellectual disability and microcephaly. He was born following a normal pregnancy with delivery at 40 weeks of gestation. His birth weight was 2445 grams (small for gestational age) and head circumference 32 cm (3rd percentile). Throughout infancy, he had failure to thrive and was underweight; however no vomiting or deterioration related to febrile illness were reported. He suffered from seizures beginning at the age of three months, for which he initially received phenobarbital and later valproic acid until the age of five years, after which his seizures spontaneously remitted. He also underwent surgery for a right inguinal hernia. He suffered from recurrent otitis media as a toddler, and at four years of age he underwent adenoidectomy and myringotomy. In childhood, he experienced walking difficulties due to progressive spasticity of his lower limbs and did not achieve independent walking. At the age of six, he underwent orthopedic surgeries for bilateral iliopsoas, adductor, hamstring, and Achilles tendon release. At no time in his development did he achieve spoken language other than a few words, but he is able to communicate with his family members using vocalized sounds. His cognitive level was evaluated as severely disabled (estimated IQ 40-50). Currently, he is able to walk with assistance albeit with a spastic gait (**Supplementary Video 1**). He is able to eat unassisted, but needs help to get dressed. At 19 years of age, magnetic resonance imaging (MRI) of the brain demonstrated mild atrophy of the cerebellum (**Figure 1B**), without

marked supratentorial abnormalities. The electroencephalogram (EEG) at age 11 was normal. Hearing and vision were not impaired, including normal fundoscopic examination and auditory event-related potentials. Echocardiography was also normal. At the most recent neurological examination (March 2016), his head circumference measured 52 cm (3rd percentile). His pupils were equal and reactive to light, but eye tracking was abnormal. No facial weakness or tongue fasciculation was observed. He did not have scoliosis, and had good control over his back. He had normal muscle strength in his upper limbs and normal deep tendon reflexes. However, supination of the upper limbs was limited, right more severely impaired than left. He had lower limb weakness and spasticity, and a foot drop (proximal muscle strength 2/5, and distal strength 1/5). Deep tendon reflexes in the lower limbs were brisk with clonus and a bilateral extensor plantar response. He had limited hip adduction and limited range of knee extension and ankle dorsiflexion bilaterally. Pain, touch, and temperature sensation was normal. Vibration test at the ankle was normal. No cerebellar signs were evident and his manual ability was normal. He had no increased urinary frequency or urgency.

The proband's 14-year-old **sister (IV-3)** also suffers from complicated spastic paraplegia, moderate intellectual disability and microcephaly. She was born after a normal pregnancy and unremarkable delivery. She presented with developmental delay evident from the age of one year old. During childhood, she experienced episodic attacks of ataxia, tremor, altered consciousness, and behavioral changes related to febrile episodes. She began walking at two years of age and spoke her first words at the age of three. She did not progress to upright ambulation until the age of three when she started limping on one leg and walking on her toes. At the age of eight, she underwent orthopedic surgeries for bilateral hamstrings and Achilles tendon lengthening. At the age of 11, she was admitted to the hospital with acute mental change and treated with methylphenidate. At present, she walks with assistance and is able to eat unassisted. She has no history of seizures. She has moderate intellectual disability (estimated IQ 50-60), with basic reading, writing and mathematics. Computerized tomography (CT) of the head (2005), repeated brain MRI (2005, 2009, 2011), and magnetic resonance spectroscopy (MRS) (2011) demonstrated unspecific isolated subcortical white matter signal abnormality in the left frontal lobe without any further grossly abnormal findings. Hearing and visual function were normal, including fundoscopic examination and auditory event-related potentials. Echocardiography was normal. Cerebrospinal fluid analysis was also normal. Blood tests including blood gases, electrolytes, lactate, ammonia, very long chain fatty acids, isotransferrin electrophoresis, amino-acids, biotinidase, thyroid functions were normal. She

underwent a liver biopsy that showed glycogen accumulation around hepatocytes, and a rectal biopsy which showed glycogen accumulation around ganglions, both of which were interpreted as normal variation. Lysosomal enzyme testing excluded metachromatic leukodystrophy (MLD), Krabbe, Tay Sachs, and GM1 gangliosidosis. At the latest physical examination (March 2016), she presented with no dysmorphisms other than bilateral syndactyly of the second and third phalanges of the feet. Both feet were in an equinovarus position. Her head circumference measured 50 cm (3rd percentile). Her pupils were equal and reactive to light, with horizontal end point nystagmus. There was no facial weakness. She exhibited stuttering speech. Hyperreflexia was evident in the upper limbs. Pronation and supination were limited in upper limbs. She had lower limb weakness, a spastic scissor gait, and walked on her toes (**Supplementary Video 2**). She also had lordosis without crouch gait. Spasticity was present in both legs with limited adduction in both hips and ankle dorsiflexion bilaterally. Deep tendon hyperreflexia as well as contralateral reflexes were present in the lower limbs, with sustained clonus and extensor plantar response. Vibration and joint position sense were reduced in the distal lower extremities. Lower limb proximal muscle strength was 3/5, distal strength was 2/5. There were no cerebellar signs, and she had no urinary frequency nor urgency.

The other two siblings of the proband (a brother of 17 years old and a sister of 15 years-old) were neurologically intact without any evidence of HSP. Neither the parents, nor any known extended family members, exhibited any of the major signs or symptoms of HSP.

Table 1 Identified exonic variants (GRCh37/hg19)

Chromosome	22	**22**
Position	37524474	**41918935**
Ref	G	**T**
Alt	A	**G**
DbSNP	rs775132140	**Na**
Gene	IL2RB	**ACO2**
Coding effect	Nonsynonymous	**Nonsynonymous**
cDNA effect	c.1318C>T	**c.1240T>G**
Protein effect	p.Pro440Ser	**p.Phe414Val**
Public database frequency		
EVS6500	Absent	**Absent**
1000G	Absent	**Absent**
HRC	Absent	**Absent**
ExAC	0.083%	**Absent**
Prediction tools		
SIFT	Tolerated	**Damaging**
Polyphen2	Benign	**Damaging**
LRT	Neutral	**Damaging**
MutationTaster	Neutral	**Damaging**
MutationAssessor	Low	**Medium**
CADD	10.29	**33**

Genetic studies

No homozygous or compound heterozygous copy number variants were identified that were shared in both affected siblings. Parametric linkage analysis under an autosomal recessive model revealed ~23 Mb of candidate genomic regions distributed across chromosomes 2, 4, 5, 17, and 22 (**Supplementary Table 1**).

Filtering of the exome sequencing data for homozygous or compound heterozygous mutations using the above-mentioned criteria revealed only two variants. The first is a homozygous variant in the gene *ACO2* (c.1240 T>G p.Phe414Val; **Table 1 and Figure 2A**). This missense variant was absent from all public databases (EVS6500, ExAC, and 1000 Genomes) and predicted to be deleterious by SIFT, PolyPhen2, Likelihood Ratio Test (LRT), Mutation Taster, and Combined Annotation Dependent Depletion (CADD) (**Table 1**) and is highly evolutionarily conserved (**Figure 2B**). Genotypes of all available subjects were confirmed by Sanger sequencing (**Figure 1A and Figure 2A**). The other segregating variant was in the *IL2RB* gene (c.1318 C>T p. Pro440Ser), with a MAF = 8 x 10^{-5} (ExAC). This variant is predicted as benign by SIFT, PolyPhen2 LRT, Mutation Taster, Mutation Assessor, and CADD (**Table 1**). Moreover, IL2RB displays very low expression levels across all developmental ages in human brain (www.brainspan.org).

Discussion

We performed genome-wide linkage analysis and exome sequencing in a consanguineous family with 2 siblings affected by hereditary spastic paraplegia, complicated by intellectual disability and microcephaly. This led to the identification of two homozygous missense variants as candidate disease-causing in this family. Based on the existing literature, absence of the variant in public databases, functional *in-silico* prediction as pathogenic, and evolutionary conservation we deem the c.1240 T>G p.Phe414Val in *ACO2* causative. *ACO2* encodes the protein aconitase 2, a critical enzyme in the tricarboxylic acid cycle (TCA) cycle. Through its enzymatic function, aconitase 2 catalyses the isomerization of citrate to isocitrate. The TCA cycle is the primary source of cellular metabolic energy, and therefore evolutionarily strongly conserved. TCA cycle enzymopathies have been described before as the cause of a variety of cerebral encephalopathies, often with muscular hypotonia and developmental delay as prominent presenting features [10].

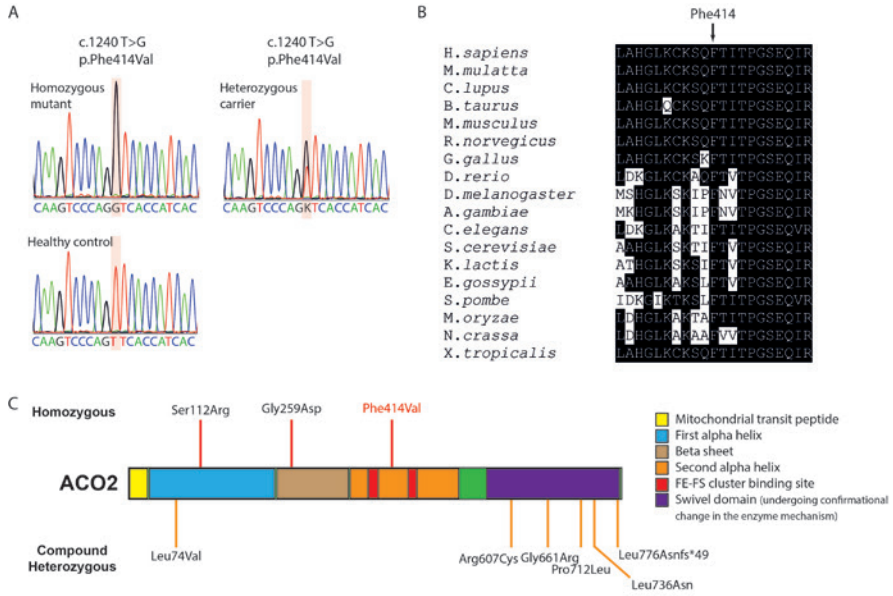


Fig 2: Sanger sequencing, conservation and summary of known *ACO2* mutations

A) Electropherograms indicating the homozygous *ACO2* mutation (affected family members), the heterozygous mutation (both parents and unaffected sibling), and the reference sequence (unaffected, unrelated subject). **B)** Amino acid conservation within the ACO2 protein homologs across species. **C)** Homozygous (top) and compound heterozygous (bottom) ACO2 mutations identified so far in patients with neurodegenerative phenotypes.

The corticospinal tract contains the longest motor neurons of the human body, which poses a significant metabolic demand on cells that require very long-distance transport of proteins and organelles to distal axon terminals. Mutations in many genes responsible for the functional integrity of this complex machinery, including mitochondrial proteins, have been implicated in different forms of HSP [1]. Our findings of the *ACO2* mutation associated with HSP provides further support for the central role of mitochondrial defects in the pathogenesis of these diseases. Although we cannot exclude that the c.1318C>T p.Pro440Ser variant in *IL2RB* plays a role in the phenotype, the brain expression levels, functional prediction as benign, and evolutionary conservation make this very unlikely.

Homozygous and compound heterozygous mutations in *ACO2* have been previously associated with varying combinations of cerebellar ataxia, retinopathy, and developmental delay (**Figure 2C and Table 2**). A p.Ser112Arg missense mutation was first identified in two

families with infantile cerebellar-retinal degeneration [11]. Affected individuals exhibited developmental delay including severe psychomotor retardation, with an age of onset between two and six months of age. Brain MRI revealed cerebellar degeneration and white matter abnormalities (dysmyelination). Optic atrophy and retinal degeneration was readily identifiable in the setting of progressively severe visual impairment. Mitochondrial aconitase enzymatic activity in lymphoblasts was significantly reduced.

Through exome sequencing of sporadic cases of complicated optic neuropathy, four patients with homozygous or compound heterozygous mutations in *ACO2* were identified. Two patients from one family had a compound heterozygous mutation (p.Leu74Val and p.Gly661Arg) and presented with decreased visual acuity in childhood with progression of ophthalmological symptoms into the fourth decade of life. Brain MRI was not performed. A third patient (homozygous p.Gly259Asp mutation) was born with a low APGAR score, intermittent episodes of central apnea, and moderate cerebellar atrophy. The fourth patient (compound heterozygous mutation, p.Leu736Asn and p.Leu776Asnfs*49) exhibited ophthalmological impairments with developmental delay and moderate cerebellar atrophy. Mitochondrial aconitase enzymatic activity was significantly reduced in all patients' fibroblasts compared to unaffected controls [12].

Last, a three-year-old sporadic patient with developmental delay, cerebellar dysfunction, and a mild auditory neuropathy in the absence of retinal degeneration was reported with compound heterozygous mutations in *ACO2* (c.1819C>T p.Arg607Cys and c.2135C>T p.Pro712Leu). Mitochondrial aconitase levels were significantly reduced in patient fibroblasts and isolated mitochondria [13].

The patients in the family described here developed severe HSP, complicated with microcephaly and intellectual disability, with some degree of intra-familial phenotypic variation. This variability is a well-known phenomenon in many neurogenetics disorders [14, 15].

Remarkably, and in contrast to the patients reported so far with other *ACO2* mutations, clinical cerebellar signs or evidence of retinal abnormalities were not present in our two patients described here. However, mild atrophy of the cerebellar vermis and hemispheres was present on the proband's MRI, as well as (supratentorial) white matter abnormalities in his affected sister.

Table 2 Genotypes and phenotypes of *ACO2* mutations

Mutation	Zygosity	Clinical Phenotype	Families described	Reference
Leu74Val	Heterozygous (only observed in combination with Gly661Arg)	Isolated optic neuropathy	1	12
Ser112Arg	Homozygous	Developmental delay, cerebellar ataxia & cerebellar atrophy	2	11
Gly259Asp	Homozygous	Episodes of central apnea, optic neuropathy & cerebellar atrophy	1	12
Phe414Val	Homozygous	Spastic paraplegia, complicated by microcephaly and intellectual disability; cerebellar atrophy without clinical cerebellar signs (in one sibling)	1	This paper
Arg607Cys	Heterozygous (only observed in combination with Pro712Leu)	Developmental delay, auditory neuropathy, cerebellar ataxia without optic neuropathy	1	13
Gly661Arg	Heterozygous (only observed in combination with Leu74Val)	Isolated optic neuropathy	1	12
Pro712Leu	Heterozygous (only observed in combination with Arg607Cys)	Developmental delay, auditory neuropathy, cerebellar ataxia without optic neuropathy	1	13
Leu736Asn	Heterozygous (only observed in combination with Leu776Asnfs*49)	Developmental delay, optic neuropathy & cerebellar atrophy	1	12
Leu776Asnfs*49	Heterozygous (only observed in combination with Leu736Asn)	Developmental delay, optic neuropathy & cerebellar atrophy	1	12

In conclusion, our findings nominate *ACO2* as a novel disease-causing gene for autosomal recessive, complicated HSP, and thus expand profoundly the phenotype associated with mutations in this gene. Genetic screening of *ACO2* should be considered for patients with complicated HSP in an effort to obtain a molecular diagnosis. Our findings provide also further support for a central role of mitochondrial defects in the pathogenesis of HSPs.

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Author contributions: C.G.B., Z.A., V.B. and S.A.K. designed the experiments. C.G.B., Z.A., A.F-V, M.A.T. and S.A.K. performed the family ascertainment. A.F-V, H.M. and M.W.V. performed the clinical studies. C.G.B. and W.F.J.vIJ. performed the genetic analyses. The genetic analyses were supervised by M.Q., G.J.B.,V.B. and S.A.K. C.G.B., Z.A., A.F-V, V.B. and S.A.K wrote the manuscript. The manuscript was reviewed, edited and approved by all authors.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflicts of interest: The authors report no conflict of interest

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Supplementary Table

Supplementary Table 1 Candidate genomic regions supported under an autosomal recessive model of inheritance

Chr	Start SNP	End SNP	Start (GRCh37/hg19)	End	LOD score	Size in Mb	Size in cM
2	rs1429258	rs1520344	155,390,620	156,671,343	1.92	1.28	1.46
4	rs2290405	rs12503220	946,974	2,850,142	1.92	1.90	2.56
5	rs9327065	rs2897883	117,345,425	124,245,657	1.92	6.90	4.45
17	rs7207403	rs4794558	47,210,506	53,347,953	1.92	6.14	6.87
22	rs926755	rs4822135	36,134,690	42,876,604	1.92	6.74	9.29
				Total		22.96	24.63

Part III |

Structural abnormalities and psychiatric disorders

6 |

Chapter 6 A balanced translocation disrupting *BCL2L10* and *PNLDC1* segregates with affective psychosis

Christian G. Bouwkamp[1,2], Anneke J.A. Kievit[2], Simone Olgiati[2], Guido Breedveld[2], Michiel Coesmans[1], Vincenzo Bonifati[2#], Steven A. Kushner[1#]

1 Department of Psychiatry, Erasmus University Medical Center, Rotterdam, The Netherlands

2 Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands

# These authors contributed equally

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Abstract

Affective psychoses are a group of severe psychiatric disorders, including schizoaffective disorder and bipolar I disorder, together affecting ~1% of the population. Despite their high heritability, the molecular genetics and neurobiology of affective psychosis remains largely elusive. Here, we describe the identification of a structural genetic variant segregating with affective psychosis in a family with multiple members suffering from bipolar I disorder or schizoaffective disorder, bipolar type. A balanced translocation involving chromosomes 6 and 15 was detected by karyotyping and fluorescence in-situ hybridization (FISH). Using whole-genome sequencing, we rapidly delineated the translocation breakpoints as corresponding intragenic events disrupting *BCL2L10* and *PNLDC1*. These data warrant further consideration for *BCL2L10* and *PNLDC1* as novel candidates for affective psychosis.

Introduction

Affective psychoses comprise a group of severely debilitating psychiatric disorders, including bipolar I disorder and schizoaffective disorder, together affecting ~1% of the population (Perälä et al., 2007). These disorders profoundly impact quality of life, including education, employment, and interpersonal relationships (Saarni et al., 2010). The suicide rate in patients with bipolar disorder is ~16% (Clements et al., 2013).

Bipolar disorder is well known to have a strong genetic component, with heritability estimates as high as ~0.75 (Sullivan et al., 2012). Despite this, the molecular genetic architecture of bipolar disorder remains largely unknown. There has been longstanding interest in the Mendelian genetics of severe mental illness using family-based linkage approaches (Badner et al., 2013; Baron, 2002) and genome-wide association studies (GWAS) (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011). However, replication of linkage findings in independent cohorts has generally been lacking (Lewis et al., 2003).

The emergence of next generation sequencing (NGS), such as whole-exome sequencing and whole-genome sequencing have enabled powerful family-based approaches for the identification of the genetic causes of disease. Here, we report the results of our investigations in a family with affective psychosis. Using traditional cytogenetic techniques followed by whole-genome sequencing, we identified a balanced translocation between the long arms of chromosomes 6 and 15, which disrupts the genes *BCL2L10* and *PNLDC1*, and segregates with affective psychosis.

Subjects and Methods

Participants

We ascertained a Dutch family of Caucasian ethnicity with a high incidence of affective psychosis (bipolar I disorder and schizoaffective disorder, bipolar type), compatible with an autosomal dominant pattern of disease inheritance (**Figure 1**). The participants were diagnosed using the structured interview for DSM-IV disorders (SCID-1). This study was performed in compliance with the Declaration of Helsinki and was approved by the medical-ethical committee of the Erasmus University Medical Center. All participants provided written informed consent.

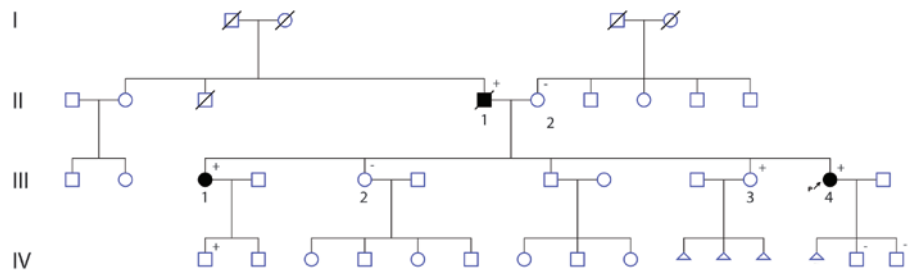

Figure 1: Pedigree

The index patient (III-4) is indicated with an arrow as the proband (P). Shaded symbols indicate family members with affective psychosis. Subjects from whom DNA was available are numbered. Plus (+) or minus (-) symbols indicate the presence or absence of the balanced translocation.

Cytogenetic studies

Karyotyping was performed on metaphase chromosomes isolated from freshly cultured peripheral blood lymphocytes from the index patient (III-4). Fluorescence in-situ hybridization (FISH) analyses were performed for the index patient (III-4) on metaphase chromosomes derived from freshly cultured peripheral blood lymphocytes according to previously published methods (Pinkel et al., 1986). Whole-chromosome-specific paints were obtained from a commercial provider (Eurodiagnostica). Probes were labelled with either biotin (Bio) or digoxigenin (Dig) using the Nick Labeling Kit (Roche). Bio/Dig were detected with a one-layer amplification step using streptavidin Alexa 594 (Molecular Probes) and anti-Dig-fluorescence (Roche). Chromosomes were counterstained with Vectashield (Vector) containing 4,6-diamidino-2-phenylindole (Sigma). Images were captured using a Zeiss Axioskop II fluorescence microscope and Genetiscan Power Gene System (Applied Imaging).

Whole-genome sequencing

Genomic DNA was isolated from peripheral blood using standard protocols. Whole-genome sequencing was performed in the index patient (III-4) at 40x average coverage depth (**Figure 1**) (Complete Genomics, Mountain View, CA, USA). After an adapter build-in step, ~400 base pair genomic fragments underwent circular PCR amplification on DNA nanoballs. Self-assembling nanoarrays with one nanoball per well were used for combinatorial probe-anchor ligation (cPAL™) to build in fluorophore-labelled dNTPs. Reads were aligned to the human reference genome version GRCh37/hg19.

Table 1 Clinical characteristics

Ped ID	II-1	II-2	III-1	III-2	III-3	III-4
t(6;15)(q26;q21) translocation carrier	Yes	No	No	Yes	Yes	Yes
DSM-IV Diagnosis	Bipolar I disorder	None	None	Bipolar I disorder	None	Schizoaffective disorder, bipolar type
Age at examination	Deceased (at age 69)	83	53	55	50	40
Age of onset depressive episodes	Unknown			16	Na	17
Number of depressive episodes				>10		3
Age of onset manic-psychotic episodes	Unknown			44	Na	23
Number of manic-psychotic episodes				3		1
Medication	Unknown	Metoprolol, barnidipine,triamterene, carbasalate calcium	Fentanyl plasters, clonidine, estriadol valerate, clonazepam	Quetiapine	None	Quetiapine
Other diagnoses	None	Hypertension, essential thrombocythemia	Fibromyalgia, chronic fatigue syndrome, anxiety symptoms	None	Three spontaneous terminations of pregnancy	Single spontaneous termination of pregnancy
Educational level	Primary school	Primary school	Secondary education	Secondary education	Higher professional education	Higher professional education

Mapped reads and coverage depth were used to identify single nucleotide variants (CNVs), small insertions and deletions (indels), copy number variants (CNVs), structural rearrangements, and mobile element insertions (Drmanac et al., 2010).

Polymerase Chain Reaction (PCR) and Sanger Sequencing

The translocation breakpoint and flanking sequence was confirmed by Sanger sequencing in the DNA samples of all ascertained family members (II-1, II-2, III-1, III-2, III-3, and III-4) (**Supplementary Methods**, **Supplementary Table 1**).

Reference sequences

The GRCh37/hg19 build was used for annotation of the whole-genome sequencing data, design of the Sanger sequencing primers, and Sanger sequence analysis. All variants identified were annotated according to GenBank reference sequences with accession numbers for *BCL2L10* (NM_020396) and *PNLDC1* (NM_173516).

Results

The phenotypic profile of all ascertained family members is provided in **Table 1**. The index patient (III-4) suffered from schizoaffective disorder, bipolar type with a history of manic-psychotic episodes and multiple inpatient psychiatric hospital admissions. In addition, she experienced a spontaneous termination of pregnancy at age 30. She was clinically stable on a maintenance regimen of 375 mg/d quetiapine. The index patient was the youngest of five siblings, with a brother and three older sisters. Her eldest sister (III-1) was diagnosed with bipolar I disorder and maintained on 75 mg/d quetiapine with residual depressive symptoms. The second oldest sister (III-2) suffered from fibromyalgia, chronic fatigue syndrome, and anxiety symptoms. Their brother had no significant history of psychiatric symptoms. Her youngest sister (III-3) had multiple spontaneous abortions in the first trimester of pregnancy in the setting of clomifene therapy to induce ovulation, but no significant history of psychiatric symptoms. Their father (II-1) was deceased but reported by the family as having a diagnosis of bipolar I disorder with multiple inpatient psychiatric hospitalizations. His medical records were unavailable for independent review. Their mother (II-2) had no significant psychiatric history.

Karyotyping and FISH revealed a balanced translocation in the index patient (III-4). The translocation breakpoints were localized to cytogenetic bands at 6q26 and 15q21, with a formal

karyotype of 46,XX,t(6;15)(q26;q21) (**Figure 2**). In order to map the precise chromosomal breakpoints, we performed whole-genome sequencing using Complete Genomics technology and confirmed by Sanger sequencing (**Figure 3**). The breakpoint on chromosome 6 was located in intron 11 of *Poly(A)-specific ribonuclease (PARN)-like domain containing 1* (*PNLDC1*), which is transcribed from the forward strand t(6;15)(q26;q21)1097+1227_1097+1228. The breakpoint on chromosome 15 was located in intron 1 of the gene *B-cell lymphoma 2 like 10* (*BCL2L10*), which is transcribed from the reverse strand t(6;15)(q26;q21)538+1460_538+1461. As a consequence of this balanced translocation, the structure and expression of a single copy of both *PNLDC1* and *BCL2L10* were disrupted and predicted to result in a heterozygous loss of function.

The presence or absence of the translocation was evaluated by Sanger sequencing in all ascertained family members (**Figure 1**). Individuals II-1, III-1, and III-3 were found to carry the t(6;15)(q26;q21) translocation with the identical breakpoints and flanking sequence as the index patient (III-4). In addition to the Sanger sequencing confirmation, individuals III-1 and III-3 were also found to carry the translocation by clinical diagnostic karyotyping. Individual III-1 had two sons, of which the eldest was confirmed by clinical diagnostic karyotyping to have inherited the translocation. Neither of the two children of the index patient III-4 were found to carry the translocation by clinical genetic testing.

Discussion

We identified a balanced translocation disrupting *PNLDC1* and *BCL2L10* that segregated with affective psychosis within a family across at least two generations. Independent genetic replication will be required to definitively evaluate the association of *PNLDC1* and *BCL2L10* with affective psychosis.

BCL2L10 encodes a 204 amino acid intracellular membrane-associated BCL2 family protein which is expressed in the brain and localized to mitochondria (Zhang et al., 2001). The BCL2L10 protein functions to negatively regulate apoptosis in the mitochondrial death pathway by preventing cytochrome c release, caspase 3 activation, and mitochondrial membrane potential collapse (Zhang et al., 2001; Cory and Adams, 2002). A previous study using array-based expression analysis identified alterations of an apoptosis-related gene set in lithium-responsive patients with unipolar depression (Lowthert et al., 2012). Notably, anti-

apoptotic BCL2 family transcripts, of which BCL2L10 is a member, were up-regulated while pro-apoptotic family members were down-regulated in the lithium responsive group.

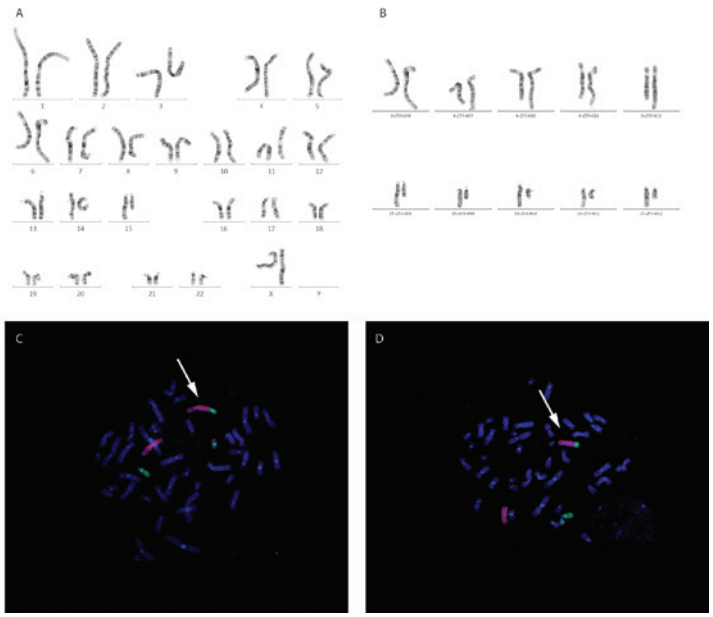

Figure 2: Cytogenetic studies

(A) Complete karyogram from subject III-4 with the inherited balanced translocation: 46,XX,t(6;15)(q26;q21).

(B) Selected karyogram images demonstrating the heterozygous abnormal representation of chromosome 6 (top row) and chromosome 15 (bottom row).

(C and D) Fluorescence in-situ hybridization showing the abnormalities in chromosome 6 **(C)** and chromosome 15 **(D)** indicated by the arrows. In **(C),** probes pertaining to chromosome 6 are labeled in red, and probes pertaining to chromosome 15 are labeled in green. In **(D),** probes pertaining to chromosome 15 are labeled in red, and probes pertaining to chromosome 6 are labeled in green. For both **(C)** and **(D)**, chromosomes are visualized in blue.

Analogously, the apoptotic regulatory function of BCL2L10 may also have contributed to the occurrence of multiple spontaneous abortions in family members carrying the *BCL2L10* disruption, as two different studies have confirmed high expression levels of this gene in human oocytes for which abnormal subcellular localization of BCL2L10 was associated with poor-quality embryos during preimplantation screening (Guérin et al., 2013; Guillemin et al., 2009; Yoon et al., 2009). However, an important and non-mutually exclusive possibility is that the spontaneous abortions are a consequence of embryos inheriting an unbalanced

translocation leading to aneuploidy as a result of meiosis involving the maternal translocation (Tharapel et al., 1985).

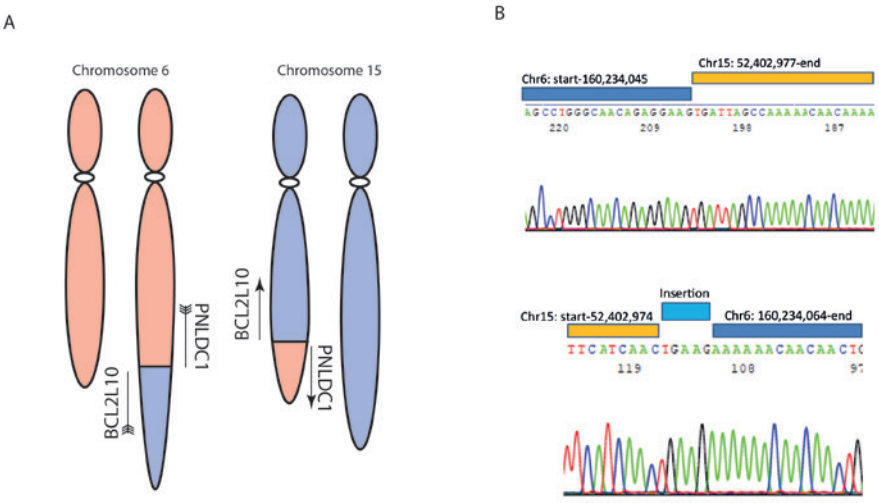

Figure 3: Chromosomal rearrangement

A) Schematic view of the balanced translocation involving chromosomes 6 and 15.

B) Electropherograms of the DNA sequence across the translocation breakpoints.

The other gene disrupted by the balanced translocation, *PNLDC1*, encodes a 520 amino acid protein containing an RNaseH-like domain and RNAse_CAF1 domain. The Poly(A)-specific ribonuclease group of proteins are involved in deadenylation of mRNA in eurkaryotes, thereby regulating mRNA levels and translation. However, the function of the nuclease-containing PARN-like protein PNLDC1 has not been investigated (Virtanen et al., 2013).

To our knowledge, there is currently no evidence for linkage with affective psychosis in the regions surrounding *BCL2L10* or *PNLDC1*, nor for common variants to be associated with affective psychosis, based on the data in the Johns Hopkins Metamoodics database for genome-wide linkage which comprises 972 families with bipolar disorder and schizoaffective disorder and GWAS data of bipolar disorder comprising 7616 cases and 10340 controls (Seifuddin et al., 2012; Pirooznia et al., 2014). In addition, the GWAS dataset from the Psychiatric Genomics Consortium of 11,974 cases and 51,792 controls provides no clear evidence of common alleles in these regions associated with bipolar disorder (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011). Furthermore, genome-wide

exome sequencing studies are not yet publically available for bipolar disorder to examine the presence of rare exonic variants in these regions.

Although schizophrenia is a form of non-affective psychosis, multiple copy number variants have been identified which increase the risk of schizophrenia and bipolar disorder (Green et al., 2016; Rodriguez-Murillo et al., 2012). Therefore, we examined the data available from the recently published Sweden Schizophrenia Exome study which involved whole-exome sequencing of 2536 patients with schizophrenia and 2543 unaffected controls (Purcell et al., 2014). In *BCL2L10*, only a single rare coding variant was identified in the entire cohort (c.467G>A, p.W156*, MAF<0.005, nonsynonymous), which was found in one patient and no controls. In *PNLDC1*, two coding variants were identified: c.13 C>T, p.R5* present in one patient and one control (within transcript NM_001271862, the effect of this variant is c.77-31 C>T), and c.244 C>T, p.L82F (NM_001271862: c.277 C>T p.L93F in) present in one patient and two controls. This scenario illustrates the difficulties in conclusively establishing a disease-causing role for very rare disease-associated variants. Longitudinal follow-up studies of this family, in addition to continued screening of other probands and case/control cohorts for rare coding variants in these genes, have the potential to provide further clarity regarding the pathophysiology of affective psychosis.

The study of patients who carry rare cytogenetic abnormalities has long been an important strategy for the identification of candidate disease-causing genes, including the first reported candidate gene for psychosis (*DISC1*) (MacIntyre et al., 2003). Although highly penetrant mutations are rare causes of psychiatric disorders, their identification have the potential to highlight molecular pathways that are mechanistically involved in disease pathogenesis among the wider group of patients who do not carry high-penetrance mutations. Prominent examples for brain diseases with an otherwise complex genetic architecture include the identification of mutations in the gene coding for amyloid precursor protein (*APP*) for Alzheimer's disease (Goate et al., 1991; Tanzi et al., 1992; Kamino et al., 1992) and the gene encoding alpha-synuclein (*SNCA*) in Parkinson's Disease and Dementia with Lewy bodies (Polymeropoulos et al., 1996). Therefore, we believe that family-based genetic studies coupled with the new generation DNA sequencing technologies hold considerable potential to contribute to the understanding of the neurobiological underpinnings of severe psychiatric illness.

Acknowledgments

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Supplementary Methods

Polymerase Chain Reaction (PCR) and Sanger Sequencing

Amplification reactions were performed in a total volume of 20 µl, containing 1x FastStart Taq DNA Polymerase buffer, 200 µM of each dNTP, 10 µM forward primer, 10 µM reverse primer (**Supplementary Table 1**), 0.5 unit FastStart Taq DNA Polymerase (Roche Diagnostics), and 20 ng genomic DNA. PCR conditions: 5' at 94°C initial denaturation followed by 10 cycles of 30" at 94°C, 30" at 70°C -10C/cycle (touch down), and 60" at 72°C, and then 25 cycles of 30" at 94°C, 30" at 60°C, and 60" at 72°C with a final extension of 5' at 72°C. 4 µl of each PCR reaction was purified using 5 units ExoI and 0.5 unit Fast AP (Fermentas) incubated for 45' at 37°C and then 15' at 80°C in a total volume of 10 µl. Direct sequencing of forward and reverse strands was performed using Big Dye Terminator chemistry version 3.1 (Applied Biosystems) as recommended by the manufacturer. Dye terminators were removed using SephadexG50 (GE Healthcare) and the remaining reaction product was loaded on an ABI 3130XL Genetic Analyzer (Applied Biosystems). Seqscape v 2.6 and Sequencing Analysis v6.0 (Life Technologies) were used for genome sequence analysis.

Supplementary Table 1: PCR Primers

Primer name	Primer sequence
Chr6_15_F	AAGCGACCTGACTGGTTTAAATGC
Chr6_15_R	ACCTGAAGAAGAGATTAGAATTGGAAAGG
Chr15_6_F	GGAATTGAACTCCAGGCTGTGG
Chr15_6_R	GCCTCTGGGCAAAGACAGACC

7 |

Copy number variation in syndromic forms of psychiatric illness: The emerging value of clinical genetic testing in psychiatry

Christian G. Bouwkamp, MSc.[1,2*], Anneke J.A. Kievit, M.D., Ph.D.[2*], Sander Markx, M.D.[1,3,4*], Joseph I. Friedman, M.D.[5,6*], Laura van Zutven, Ph.D.[2], Rick van Minkelen, Ph.D.[2], Terry Vrijenhoek, Ph.D.[7], Bin Xu, Ph.D.[3,4], Ineke Sterrenburg-van de Nieuwegiessen, M.D.[8], Joris A. Veltman, Ph.D.[9,10], Vincenzo Bonifati, M.D., Ph.D.[2], Steven A. Kushner, M.D., Ph.D.[1]

1 Department of Psychiatry, Erasmus MC, Rotterdam, The Netherlands
2 Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands
3 Department of Psychiatry and Center for Precision Neuropsychiatry, Columbia University, New York, NY, USA
4 New York State Psychiatric Institute, New York, NY, USA
5 Departments of Psychiatry, Neuroscience and Human Genetics, Mount Sinai School of Medicine, New York, NY, USA
6 Clinical Neuroscience Center, Pilgrim Psychiatric Center, W. Brentwood, NY, USA
7 Department of Medical Genetics, University Medical Center, Utrecht, The Netherlands
8 Delta Psychiatric Center, Poortugaal, The Netherlands
9 Department of Human Genetics, Donders Centre for Neuroscience, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
10 Department of Clinical Genetics, GROW - School for Oncology and Developmental Biology, Maastricht University Medical Centre
* These authors contributed equally

Manuscript submitted

Abstract

Genetic factors are a strong determinant of mental health. However, patients with psychiatric disorders are seldomly referred for clinical genetic evaluations due to their generally limited diagnostic yield. Recent advances have resulted in a substantial increase of knowledge regarding the genetic architecture of psychiatric disorders. Copy number variants (CNVs) are alterations of the genome that result in variation of the number of copies of one or more genes. CNV analysis is now widely implemented in the clinical genetic testing algorithm for a wide variety of medical conditions. Accumulating evidence has demonstrated highly penetrant CNVs in a clinically-significant proportion of patients with psychiatric disorders. Specifically, patients with syndromic forms of psychiatric illness have a particularly high rate of diagnostically-relevant CNVs. We confirm this finding in a series of 50 patients with Axis I psychiatric disorders and syndromic features, replicated across two independent cohorts. Pathogenic or likely pathogenic CNVs were identified in 24% of cases. Based on the current literature and the newly reported replication cohorts, we conclude that clinical genetic testing should be considered for patients with syndromic forms of psychiatric illness.

Background and introduction

Despite extensive efforts and recent major successes (1; 2), the genetic architecture of psychiatric disorders has remained insufficiently understood to recommend genetic testing as part of the routine diagnostic evaluation in clinical psychiatry (3). With recent developments of advanced molecular genetic techniques and the considerable reduction in costs, there has been a consequently dramatic improvement in our understanding of the genetic underpinnings of severe psychiatric disorders (1; 2; 4–6). The strong heritability of severe psychiatric disorders was initially suggested by high rates of concordance in studies of monozygotic twins as well as in adoptions studies, especially in those involving monozygotic (MZ) twins (7). Additionally, in large epidemiological studies, it has been repeatedly demonstrated that the risk of psychiatric illness is inversely proportional to the genetic distance from an affected relative (8).

Large international consortia have undertaken highly successful efforts to unravel the genetic underpinnings for several of the major psychiatric disorders. For schizophrenia (SCZ), a genome-wide association studies (GWAS) was performed using >30,000 patients and >120,000 controls which allowed for the identification of 108 loci with genome-wide significance (1). Using a polygenic risk score, these alleles account for an estimated 3.4% of the variance in the risk for schizophrenia, which is generally considered insufficient for implementation as a routine clinical diagnostic (9). An effort to identify associated rare variants in Sweden using roughly 2500 cases and an equal number of controls yielded no variants reaching genome-wide significance (4), however multiple genes have been found to exhibit an increased burden of *de novo* mutations among patients with SCZ (10; 11). For bipolar disorder (BD), a GWAS has been performed including a total of ~12,000 patients and ~52,000 controls, in which genome-wide significant associations were observed for *CACNA1C and ODZ4* (12). For major depressive disorder (MDD), no significant SNPs had been identified as of 2016 in a GWAS comprising ~9,200 cases and ~9,500 controls in the discovery phase and ~6,800 cases and ~50,000 controls in the replication phase (13). However, recently 15 genetic loci have been published to be associated with the risk of MDD in a study that looked at ~75,000 patients with self-reported MDD and ~231,000 controls. Suggestively associated SNPs were brought forward to a replication phase with ~45,000 self-reported MDD patients and ~106,000 controls. The polygenic risk score was also associated with secondary phenotypes such as taking antidepressant medication, medication for mental health and anxiety (14).

Genetic studies of autism spectrum disorder (ASD) have yielded notably strong associations with rare monogenic syndromes, including Fragile X syndrome, Tuberous Sclerosis Complex, and Angelman Syndrome (15). Recent genetic studies investigating idiopathic autism spectrum disorder, epilepsy, and intellectual disability focused on identifying *de novo* mutations (16). The most recent study, involving 2500 families, found that *de novo* coding mutations and copy number variants (CNVs) together explain approximately 30% of simplex ASD cases (2). CNV analysis confirms known risk loci for ASD in the same cohort (1q21.1, 3q29, 7q11.23, 16p11.2, 15q11.2-13, and 22q11.2) (17). Similarly, *de novo* point mutations and CNVs were recently shown as the major cause of severe intellectual disability (18). Interestingly, a recent study demonstrated a shared genetic vulnerability among patients with *de novo* mutations in their predisposion to a confluence of neurodevelopmental abnormalities, congenital heart disease, and extracardiac congenital anomalies (19) providing further evidence of pathogenic rare genetic variation in syndromic forms of psychiatric illness.

Copy Number Variants (CNVs)

Over the past decade, increasing attention has been focused on alterations in gene dosage through CNVs resulting in deviations from the diploid state (20; 21) **(Figure 1)**. CNVs are a common source of genetic variation with an average of 1.44 CNVs per individual and an average size of ~205 Kb (22). However, a wide variety of CNVs have been shown to be an important source of pathogenic mutations, particularly larger variants (18; 23; 24), including insertions, deletions, and duplications **(Figure 1)**. With the advent of microarray technology, reliable detection of CNVs has been widely implemented in a standardized and relatively low cost workflow.

Rare CNVs have been well demonstrated to be enriched in patients with a variety of severe psychiatric disorders (5; 25; 26). Rees and colleagues recently studied a large cohort of patients with SCZ (n=6882) and healthy controls (n=6316). Overall, patients with SCZ were significantly more likely to carry relatively large and rare CNVs (>10 Kb in size with a population frequency <1%; 2.48% in SCZ patients versus 0.92% in controls, $P = 1.46 \times 10^{-12}$) (5). When combined with previous CNV analyses of case-control cohorts for SCZ, significant SCZ-associated CNVs were identified at multiple loci, including 1q21.1, *NRXN1*, 15q11.2, 16p11.2, 22q11.2, and the Angelman/Prader-Willi syndrome locus (5; 27). Remarkably, the 22q11.2 microdeletion was observed in 0.29% (n=56/19,084) of patients, but absent in 77,055 controls ($P = 4.4 \times 10^{-40}$). Carriers of these rare pathogenic CNVs have a significant increase in

the risk to develop psychosis and a range of other neuropsychiatric disorders including mood- and anxiety disorders, attention deficit hyperactivity disorder (ADHD), ASD (28), Parkinson's disease (29), as well as a variety of congenital malformations (30).

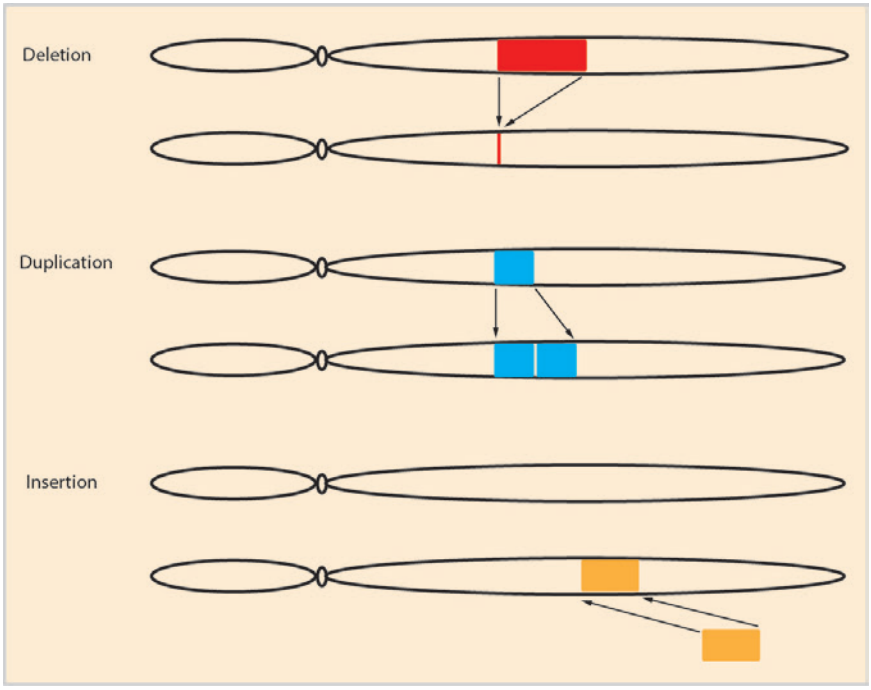

Figure 1. Schematic of CNVs. Segments of DNA can be deleted, duplicated, or inserted. Here, a duplication is shown in tandem, but duplications can also be located in more distant parts of the same chromosome or on other chromosomes, thereby potentially disrupting other genes.

The Psychiatric Genetics Consortium recently published the results of a SCZ case/control study of CNVs in the Swedish population (4719 cases, 5917 controls). Patients with SCZ had a significantly higher burden of rare (<1% general population frequency) CNVs compared with controls, most notably deletions at 3q29 and 22q11.2, and duplications at 16p11.2 and 17q12. However, only 0.14% of the variance in the risk for SCZ was explained by these rare CNVs, while ~3.4% of the variance was explained by the common variant polygenic risk (31). Taken together, the relatively low prevalence of highly penetrant CNVs among patients with non-syndromic SCZ currently appears to limit the cost-effectiveness of CNV testing in the psychiatric diagnostic workup, however with increasingly large datasets and continued reductions in cost this remains a distinct possibility in the future.

In comparison to patients with non-syndromic forms of psychiatric illness, those with syndromic features have a significantly higher frequency of CNVs. In one of the first studies

to examine CNVs in patients with syndromic psychiatric illness, deletion of *CNTNAP2* was found to be associated with co-morbid SCZ and epilepsy (32). A subsequent study described patients with co-morbid SCZ and epilepsy, in which 4% of the cases had a 15q11-q13 duplication while none were found in controls (33). Moreover, in a study investigating the relative frequency of CNVs in SCZ patients with or without intellectual disability, an excess of 15q11.2 duplications and deletions >1Mb was found in patients with SCZ and co-morbid intellectual disability. A large duplication detected in one patient with a 15q11.2 duplication was found to independently segregate in a large family with SCZ and co-morbid intellectual disability, hearing impairment, and ophthalmological problems (retinitis pigmentosa and/or cataracts) (34). Moreover, patients with childhood-onset (<13 years old) SCZ have a significantly higher burden of pathogenic CNVs than patients with an age of onset ≥13 years old (35).

The clinical genetic examination and consultation

Clinical geneticists provide a diagnostic service and counseling for individuals or families with, or at risk for, conditions that may have a discernible genetic etiology. Ideally, a clinical genetics consultation for patients with severe psychiatric disorders would be an opportunity to assess etiology, prognosis, and risk for offspring through probability estimates based on empirical genetic findings (for example, see **Table 1** regarding median recurrence rates for SCZ). In certain instances, clinical, molecular, or metabolic diagnostics can provide insight into the genetic cause of the disorder or syndrome.

When a patient is referred for genetic consultation, the proband, parents and/or spouse are typically interviewed to learn about the motivation for their visit. In addition to the anamnesis, a detailed family history is obtained. Furthermore, information from the parents of the proband is collected regarding the pregnancy, delivery, and early development. Existing relevant medical records are requested of patients and affected relatives after consent. In addition, the clinical geneticist performs a physical and dysmorphology examination of the proband in order to identify any signs that may indicate a syndromic and/or genetic disease presentation, usually referred to as minor physical anomalies (MPAs). MPAs are congenital anatomical defects (e.g., deformities of the head, eyes, ears, mouth, palate, hands and feet) thought to be indicative of abnormal ectodermal development during the first and/or second trimester (36).

Table 1. Recurrence rate x genetic distance for schizophrenia. Adapted from Austin et al. 2006

Degree	Relationship to proband	Median Risk (%)
1st degree	Parent	5.6
	Child with 1 affected parent	13
	Child with 2 affected parents	46
	Sibling	9
	Sibling with 1 affected parent	16.85
	Monozygotic twin	48
	Dizygotic twin	13.5
	Half-sibling	4.6
2nd degree	Grandchild	4.5
	Uncle/Aunt	3
3rd degree	Niece/Nephew	4
	Cousin	1.75

Since the central nervous system (CNS) is of ectodermal origin, MPAs are generally viewed as potential indicators of abnormal CNS development (37). A large number of studies have documented an increased prevalence of MPAs in patients with psychiatric disorders of a presumed neurodevelopmental etiology, such as SCZ or ASD (38).

In the current study, we specifically focused our investigation on patients with a syndromic form of psychiatric illness which we define as: involving an Axis I disorder, in combination with intellectual disability and/or congenital anomalies. A total of 50 patients with syndromic psychiatric illness were screened for CNVs, comprised of two independent prospectively recruited consecutive case series. In total, we identified pathogenic or likely pathogenic CNVs in 24.0% of patients (95% CI: 12.2 to 35.8%), with analogous findings in each independent cohort.

Case series

Pilgrim Psychiatric Center

Genetic testing, in combination with psychiatric, medical, and neuropsychological assessments were performed in 19 consecutive patients receiving chronic inpatient psychiatric care at Pilgrim Psychiatric Center (PPC) who presented with syndromic forms of SCZor schizoaffective disorder (**Table 2**).

Methods

Patients: This study was approved by the institutional review boards (IRBs) of Pilgrim Psychiatric Center, Brentwood, NY and the Mt. Sinai School of Medicine, New York. Patients who received no prior genetic testing were evaluated between February 2000 and June 2001. Informed consent was obtained from all patients. Patients with SCZor schizoaffective disorder were diagnosed according to DSM-IV-TR criteria based on data obtained from the Comprehensive Assessment of Symptoms and History (CASH) (39). All patients received neuropsychological assessment utilizing the Wechsler Adult Intelligence Scale – Third Edition (WAIS-III). Review of medical charts and family history, dysmorphology assessment and physical examination were performed by J.I.F. and S.M.

Genotyping: DNA obtained from venous whole blood-isolated leucocytes was genotyped using the Affymetrix Human GeneChip Human Mapping 250K Nsp Assay, which contains 250,00 SNPs. All sample processing was completed at Mt. Sinai School of Medicine according to manufacturer's protocol. Following hybridization, we used the Affymetrix GeneChip Command Console (AGCC; Affymetrix, Santa Clara, CA) to acquire raw genotyping data, perform quality control analysis, and genotype calling The coordinates of all CNVs were based on the human genome NCBI build hg18. Deletions were considered if supported by more than 5 subsequent probes and larger than 150 Kb in size, or if known to be associated with a relevant phenotype. Duplication were considered if supported by more than 7 subsequent probes and larger than 200 Kb in size, or if known to be associated with a relevant phenotype. The results were filtered for common CNVs (population frequency >1%) present in the UCSC genome browser (hg18), Database of Genomic Variants, and in-house database. Copy-neutral regions of homozygosity (ROHs) were considered if larger than 5Mb. Classification of CNV pathogenicity was made according to the clinical guidelines of the American College of Medical Genetics (40). CNVs were classified as pathogenic if there were at least two published articles in the literature describing these variants. All other CNVs were classified as variants of uncertain significance (VOUS), but with the additional specifier "of likely pathogenicity" if genes in the CNV region were known to be relevant to neurodevelopment and brain physiology based on genetic and functional evidence derived from published papers in the literature.

Results

Demographic characteristics are described in **Table 2**. The sample consisted of a similar proportion of males (47%) and females (53%). All patients had either SCZ (79%) or schizoaffective disorder (21%), in addition to intellectual disability ranging from borderline/mild to moderate/severe. Pathogenic or likely pathogenic CNVs were found in 5 of 19 patients (26.3%). An additional 5 patients had variants of unknown significance (VOUS) (**Supplementary Information**). Genomic coordinates of the identified CNVs are provided in **Table 3**. Detailed characteristics of these 10 patients regarding dysmorphic features, intelligence testing and psychiatric diagnoses are reported in **Table 4**.

Table 2. Patient characteristics for cohort referred for clinical genetic counselling

Pilgrim Psychiatric Center		
Gender	9 males and 10 females	
Age	38.4 (SD 10.0)	
Primary psychiatric diagnosis	Schizophrenia	15
	Schizoaffective disorder	4
Erasmus MC		
Gender	20 males and 11 females	
Age	34.6 (SD 11.7)	
Primary psychiatric diagnosis	Autism spectrum disorder	10
	Schizophrenia	6
	Psychotic disorder NOS	5
	Schizoaffective disorder, bipolar type	4
	Unipolar mood disorder	2
	Bipolar disorder	1
	Attention deficit hyperactivity disorder	1
	Behavioral disorder NOS	1
	Impulse control disorder	1
Family history	Positive	23
	Negative	8

Case descriptions of patients with pathogenic or likely pathogenic CNVs:

Patient PPC-3 was a 52 year-old male diagnosed with SCZ with an age of onset of 14 years-old. His medical history was notable for hyperlipidemia and sideroblastic anemia. The patient exhibited dysmorphic features including an elongated face with narrow orbital fissures, hypertelorism, flat nasal bridge, malar flatness, and retrognathia. Moreover, he developed a late-onset seizure disorder at age 59. He completed secondary school but was never employed and never married. His illness course was chronic and unremitting with increasingly severe cognitive impairment and regressive behavior. WAIS III testing at age 52 revealed a verbal IQ (VIQ) of 79, a performance IQ (PIQ) of 68, and a full-scale IQ (FSIQ) of 77. CNV screening revealed a 2Mb duplication of the long arm of chromosome 22 (22q11.21). 22q11.2 microduplications have been associated with both SCZ and ASD (41). This variant is classified as a **pathogenic variant**. The patient was also noted to have a deletion involving

Table 3. Genomic coordinates (hg18) of the CNVs identified in the cohorts

Pilgrim Psychiatric Center		
Patient	**Variant**	**Genomic coordinates (hg18)**
PPC-2	Dup	Chr22:48,114,921-48,458,127
PPC-3	Del	Chr7:145,582,575-145,741,967
PPC-3	Dup	Chr8:133,398,572-133,821,537
PPC-3	Dup	Chr22:16,615,108-18,594,783
PPC-7	Dup	Chr15:58,705,395-58,764,487
PPC-9	Dup	Chr6:124,846,876-125,239,117
PPC-9	Dup	Chr7:149,198,031-149,883,752
PPC-9	Dup	Chr9:132,351,166-132,715,588
PPC-9	Dup	Chr10:128,282,537-129,138,470
PPC-10	Dup	Chr1:212,247,344-212,557,152
PPC-10	Del	Chr22:17,275,227-19,790,008
PPC-11	Dup	Chr16:18,028,700-20,139,336
PPC-12	Del	Chr7:146,360,344-147,928,119
PPC-15	Dup	Chr4:4,511,826-4,748,685
PPC-16	Dup	Chr6:162,807,280-163,306,684
PPC-18	Dup	Chr2:45,303,454-45,813,464

Erasmus MC		
Patient	**Variant**	**Genomic coordinates (hg18)**
EMC-1	Del	Chr5:37,423,970-37,662,323
EMC-4	Dup	Chr7:0-481,295
EMC-9	Del	Chr3:113,618,774-116,995,580
EMC-10	Del	Chr1:144,959,767-146,292,125
EMC-11	Dup	Chr11:107,153,898-107,489,611
EMC-11	Dup	ChrX:87,540,740-87,757,911
EMC-12	Dup	ChrY:0-2,736,035
EMC-12	Dup	ChrY:154,587,409-154,913,754
EMC-12	Dup	ChrY:0-27,198,031
EMC-14	Del	Chr16:29,528,999-30,171,562
EMC-16	Dup	Chr3:836,618-1,438,346
EMC-16	Dup	Chr11:50,080,786-50,675,483
EMC-21	Dup	Chr5:99,785,428-100,021,429
EMC-30	Del	Chr13:106,234,020-107,960,598
EMC-31	Del	Chr3:2,226,296-2,292,563
EMC-31	Dup	Chr3:2,341,695-2,571,325
EMC-31	Del	Chr8:82,798,696-83,515,397

CNTNAP2. However, it concerns a 159 Kb intronic deletion in *CNTNAP2* which does not involve any coding sequence and is therefore classified as **VOUS**. Lastly, we identified a 423 Kb duplication on the long arm of chromosome 8 (8q24.22) containing *KCNQ3, LRRC6*, and *TMEM71*. KCNQ3 encodes the voltage-gated potassium channel, subfamily Q, member 3. Heterozygous missense mutations in this gene have been associated with seizures of the benign neonatal subtype, type 2 (42–44). The protein is mainly expressed in the human brain. LRRC6 is involved in autosomal recessive primary ciliary dyskinesia (45; 46). TMEM71 has not been implicated with this phenotype. Based on the gene content, this variant is classified as **VOUS; likely pathogenic**, especially in relationship to the seizure disorder.

Patient PPC-7 was a 35 year-old female diagnosed with schizoaffective disorder with an age of onset of 19 years-old. She was noted to also have a seizure disorder, mitral valve prolapse and tricupsid insufficiency as measured by echocardiogram, migraine headaches, and strabismus, as well as dysmorphic facial features including narrow orbital fissures, a square nasal root with hypoplastic alar nasi, and retrognathia. The patient competed 12 years of

special education, was never married and had a chronic, disabling course of illness. WAIS III testing revealed a VIQ of 64, a PIQ of 51, and a FSIQ of 54. CNV screening identified a 580 Kb genomic duplication at chromosome 15q22. The breakpoints were fine-mapped to the first two exons of isoform 1 of the retinoid-related orphan receptor alpha (*RORa*) gene. *RORa* has four known isoforms, of which the affected exons are unique to isoform 1 which is specifically expressed in the brain (47). Homozygous deletion of the murine homolog, *rora*, leads to Purkinje cell degeneration and severe ataxia (48). Human *RORa* functions during neurodevelopment and has been associated with a range of neuropsychiatric disorders, including MDD, BD, and ASD (49–51). This variant is classified as **VOUS; likely pathogenic**.

Patient PPC-9 was a 21 year-old female diagnosed with schizoaffective disorder. Age of onset was in her second decade, which was followed by multiple subsequent inpatient admissions due to a chronic remitting/relapsing course, culminating in long-term hospitalization at Pigrim Psychiatric Center. She had a known history of self-injurious behavior. She was of short stature with a medical history notable only for a pilonidal cyst. Intelligence testing revealed a WAIS III verbal IQ of 65, a performance IQ of 58 and a full-scale IQ of 59. Genetic testing revealed four large rare CNVs: 1) a 392 Kb duplication on the long arm of chromosome 6 (6q22.31) affecting *TCBA1*, which has been implicated as a candidate region for SCZ (OMIM #181500) as well as ASD (52). This variant is classified as **VOUS; likely pathogenic**. 2) A 364 Kb duplication on the long arm of chromosome 9 (9q34.11-34.12). 9q34 duplication syndrome has been associated with many neuropsychiatric disorders, such as ASD and intellectual disability (53). The duplication affects *NKAIN2*, a gene that has been previously associated with SCZ (54). This variant is also classified as **VOUS; likely pathogenic**. 3) A 686 Kb duplication on the long arm of chromosome 7 (7q36.1) containing *LRRC61, RARRES2, GIMAP8, GIMAP7, REPIN1, ZNF775, ZBED6CL*, and *ATP6VOE2*. None of these genes are known to be associated with the phenotype. This variant is classified as a **VOUS**. 4) A 856 Kb duplication on the long arm of chromosome 10 (10q26.2) containing *DOCK1* and *FAM196A*. None of these genes have been implicated with the phenotype. This variant is classified as a **VOUS**.

Patient PPC-10 was a 27 year-old female diagnosed with a severe, deteriorating course of SCZ (age of onset unknown). She received special education and completed secondary school.

She was never married and never employed. Her medical history included the surgical repair of a congenital cleft palate, velopharyngeal insufficiency with hypernasal voice, chronic middle ear infections, recurrent episodes of pneumonia, short stature, esotropia, aortic insufficiency, and tricuspid regurgitation. The patient has a seizure disorder with an abnormal background EEG showing bi-frontal and bi-temporal slowing. She exhibited prominent dysmorphic features, including narrow orbital fissures, telecanthus, square nasal root, bulbous/prominent nose, retrognathia. WAIS III testing revealed a VIQ of 66, a PIQ of 59, and a FSIQ of 60. Microarray screening revealed a 2.6Mb deletion on the long arm of chromosome 22 (22q11.21), a well-established CNV associated with the 22q11.2 Deletion Syndrome (OMIM #188400). 22q11.2 microdeletions are currently the most well-established genetic risk factor for SCZ (26). This variant is classified as **pathogenic**. In addition, we identified a 310 Kb duplication on the long arm of chromosome 1 (1q41) containing *PROX1* and *SMYD2*. These genes have not been previously associated with the phenotype. This variant is classified as a **VOUS**.

Patient PPC-12 was a 50 year-old female diagnosed with SCZ, the symptoms of which first manifested at age 18. She attended high school until the 10th grade, was never married and never employed. Her medical history was notable for seizure disorder, unilateral exotropia, and strabismus. WAIS III testing revealed a VIQ of 66, a PIQ of 65, and a FSIQ of 63. She was assessed by the WRAT-R Reading subtest to estimate a pre-morbid verbal IQ, and was found to have a premorbid full-scale IQ of 94, suggesting a trajectory of cognitive decline following the onset of SCZ. The patient also experienced a significant deterioration in social and executive functioning leading to severely poor self-care skills requiring full assistance with activities of daily living. Her mother and both of her mother's siblings suffered from severe psychiatric illnesses that also required long-term hospitalization. At age 34, she experienced her only documented seizure, characterized as generalized tonic-clonic. Electroencephalography (16 channel) demonstrated slow dysrhythmia in theta with occasional delta waves, predominantly localized to the frontal lobes. Brain MRI demonstrated mild cerebral atrophy. Microarray screening identified a heterozygous 1.57Mb deletion at the 7q35–7q36.1 locus which includes *CUL1*, *EZH2*, *PDIA4* and *CNTNAP2* (32). *CNTNAP2* encodes contactin-associated protein-like 2 (CNTNAP2) which regulates neuronal-glial interactions and neural cell migration (55; 56). Both CNV deletions and exonic mutations of CNTNAP2 have been previously associated with SCZand ASD (57; 58). This variant is classified as **pathogenic**.

Erasmus University Medical Center

Clinical genetic, medical, and psychiatric assessments were performed in 31 consecutive patients receiving chronic inpatient psychiatric care who presented with syndromic forms of Axis I psychopathology including mild-to-moederate intellectual disability, multiple congenital abnormalities, or dysmorphic features..

Methods

Patients: This study was approved by the institutional review board of the Erasmus University Medical Center, Rotterdam, The Netherlands. Patients were referred by their treating psychiatrist to the Erasmus MC Department of Clinical Genetics for diagnostic evaluations between January 2012 and July 2013. All patients were diagnosed according to DSM-IV-TR using the Structured Clinical Interview for DSM-IV Axis I Disorders. Family history, dysmorphology assessment, and physical examination were performed by a clinical geneticist (A.J.A.K.). Family members were invited to participate in the clinical genetic consultation. Unfortunately in all cases but one, family members declined participation.

Genotyping: Purified genomic DNA was hybridized to Illumina HumanCytoSNP-12v2.1 microarrays (Illumina, San Diego, CA, USA) allowing for an average resolution of 300 Kb over the genome. The arrays were analysed using Illumina iScan Control and Genome studio v2.1 software, as well as Nexus Copy Number Discovery v5.0 software (Biodiscovery, Hawthore, CA, USA). Deletions were considered if supported by more than 5 subsequent probes and larger than 150 Kb in size, or if known to be associated with a relevant phenotype. Duplications were considered if supported by more than 7 subsequent probes and larger than 200 Kb in size, or if known to be associated with a relevant phenotype. The results were filtered for common CNVs (population frequency >1%) present in the UCSC genome browser (hg18), Database of Genomic Variants (DGV), and in-house database. Copy-neutral regions of homozygosity (ROHs) were considered if larger than 5Mb. For classification of CNV pathogenicity (pathogenic, variant of uncertain significance (VOUS), and VOUS with additional annotation of likely pathogenicity), we adhered to the guidelines of the American College of Medical Genetics (40). For Fragile X testing, *FMR1* trinucleotide repeat expansion was screened by Southern Blot.

Table 4. Characteristics for patients with positive genetic findings Pilgrim Psychiatric Center

Pat nr.	Gender	Age	Psychiatric disorder	FSIQ	PIQ	VIQ	Educational level	Somatic symptoms	Facial & body characteristics	Microarray results	Diagnostic classification	ROH
PPC-2	Male	32	Schizophrenia	75	79	75	11th grade education	MRI findings, psychogenic polydipsia	Elongated face, cleft palate, pectus excavatum	dup22q13.33 (343Kb)	VOUS	None
PPC-3	Male	52	Schizophrenia	77	68	79	Graduated high school	Hyperlipidemia, sideroblastic anemia, late-onset seizure disorder (age 59)	Elongated face, narrow orbital fissures, hypertelorism, flat nasal bridge, malar flatness, retrognathia	del7q35 (159Kb), dup8q24.22 423Kb), dup22q11.21(2Mb)	Pathogenic	None
PPC-7	Female	35	Schizoaffective disorder	54	51	64	Special education (12 years)	Seizure disorder, mitral valve prolapse and tricuspid insufficiency, strabisumus, migraine headaches	Narrow orbital fissures, square nasal root with hypoplastic alae nasi, retrognathia	dup15q22(580Kb)	VOUS; likely pathogenic	None
PPC-9	Female	21	Schizoaffective disorder	59	58	65	Unknown	Pilonidal cyst	Short stature	dup6q22.31 (392Kb), dup7q36.1(686Kb), dup9q34.11-34.12 (364Kb), dup10q26.2(856Kb)	VOUS; likely pathogenic	None
PPC-10	Female	27	Schizophrenia	60	59	66	Completed secondary school; special education	Cleft palate, seizure disorder, velopharyngeal insufficiency, chronic middle ear infections and recurrent episodes of pneumonia, eotropia, aortic insufficiency and tricuspid regurgitation	Narrow orbital figures, telecanthus, square nasal root, retrognathia, prominent/bulbous nose, short stature	dup1q41 (310Kb), del22q11.2 (2.5Mb)	Pathogenic	None
PPC-11	Male	40	Schizophrenia	64	65	68	Special education	None	Sloping forehead, protruding supraorbital ridges, square nasal root, wide nasal tip, short philtrum, retrognathia, malar flatness, short and broad sternum, cleft palate	dup16p12.3 (2.11Mb)	VOUS	None
PPC-12	Female	50	Schizophrenia	63	65	66	Dropped out of secondary school	Seizure disorder,unilateral exotropia, strabismus	None	del7q35-36.1(1.57Mb)	Pathogenic	None
PPC-15	Female	42	Schizophrenia	61	69	61	9th grade education	Psychogenic polydipsia, hyponatremia. Seizures secondary to hyponatremia, MRI findings	High nasal root protrusion, kyphosis	dup4p16.3-16.2 (237Kb)	VOUS	None
PPC-16	Male	48	Schizophrenia	Could not be performed			Graduated high school	Mitral valve prolapse with mitral regurgitation, seizures after clozapine treatment	Narrow orbital fissures, retrognathia, protruding supra-orbital ridges, pectus excavatum	dup6q26 (499Kb)	VOUS	None

Pat nr.	Gender	Age	Psychiatric disorder	FSIQ	PIQ	VIQ	Educational level	Somatic symptoms	Facial & body characteristics	Microarray results	Diagnostic classification	ROH
PPC-18	Male	36	Schizoaffective disorder	90	83	92	Graduated high school	Hypothyroidism, psychogenic polydipsia, hyponatremia, chronic neutropenia, hiatal hernia, obesity	High nasal root protrusion, flat nasal alae, diminished vermillion of the upper lip, clefted nasal tip, mild midine cleft lower lip, hypoplastic teeth, hypoplastic earlobes, and slender tapered fingers, high-arched V-shaped steepled cleft palate	dup2p21 (510Kb)	VOUS	None

FSIQ Full-scale IQ; PIQ performance IQ; VIQ verbal IQ; ROH Regions of homozygosity

Metabolic studies: Blood plasma fraction was isolated according to standardised clinical protocols from whole blood collected in lithium-heparin coated blood tubes and urine was collected using sterile urine containers. Basic metabolic diagnostics was performed for the following metabolites: acylcarnicites, amino acids, bile acids, creatin, guanidinoacetate, homocysteine, homogentisic acid, imidazole compounds, methylmalonic acid, mucopolysaccharides, oligosaccharides, organic acids, orotic acids, phenylalanine, tyrosine, phytanic and pristanic acid, purines and pyrimidines, sialic acid, sialotransferrins, sugars and sugaralcohols, sulfatides, tetraglucoside, and very long chain fatty acids.

Results

The majority of patients were male (64.5%, 20/31), with the majority of Axis I disorders either ASD (32.3%, 10/31) or a psychotic disorder (48.4%, 15/31) (**Table 2**). Pathogenic or likely pathogenic CNVs were found in 7 of 31 patients (22.6%). An additional 4 patient has variants of unknown significance (VOUS) (**Supplementary Information**). The genomic coordinates of the identified CNVs are provided in **Table 3**. The detailed characteristics of these 11 patients regarding dysmorphic features, psychiatric diagnoses, and family history are described in **Table 5**. Metabolic screening of blood plasma and urine, as well as Fragile X testing was normal for all patients included in this cohort.

Case descriptions of patients with pathogenic or likely pathogenic CNVs:

Patient EMC-9 was a 53-year old male and had a diagnosis of dysthymic disorder and mild intellectual disability with a total IQ of 55. His social emotional development was estimated to be that of a 6 year-old. He exhibited an elongated face, ptosis, narrow palpebral fissures, a large, pear-shaped nose, and central obesity. We found a 3.38Mb deletion on the long arm of chromosome 3 (3q13.2-q13.31) that includes 25 genes. Molin and colleagues describe several patients with a 3q13.31 microdeletion syndrome (59; 60). The 3q13.31 microdeletion syndrome is associated with developmental delay, muscular hypotonia, hypoplastic male genitalia, characteristic facial features, and obesity. This variant is classified as **pathogenic**.

Patient EMC-10 was a 22-year old male and had a diagnosis of PDD-NOS and intermittent explosive disorder with mild intellectual disability (Full-Scale IQ 69). His social-emotional development level was that of a 3-7 year-old. We identified a 1.33Mb deletion on the long arm of chromosome 1 containing the critical region of the 1q21.1 deletion syndrome, a risk allele

with a variable phenotype including developmental delay, hypotonia, microcephaly, cardiac abnormalities, hypermobility, and dysmorphisms (61). Our patient did not show notable dysmorphic features, except for hypoplastic alae nasi. This variant is classified as **pathogenic**.

Patient EMC-11 was a 27-year old male and had a diagnosis of PDD-NOS and dysthymic disorder. He had a low average total IQ of 85 with clinodactily of the 5th finger, a hemangioma, and striae on arms and abdomen. He carried 2 duplications: one on the long arm of chromosome 11 (11q22.3) containing *SLC35F2*, *RAB39A*, and *CUL5* and one on the long arm of chromosome X (Xq21.31) containing the ncRNA M90754. No other patients with these duplications have been described in the literature. A *de novo* mutation in *CUL5* was identified in a patient with ASD (62). The protein product of *CUL5*, Cullin5, interacts with Dab1 to regulate cortical neurodevelopment (63). Their respective family members, *RAB39B* and *CUL3*, have been also implicated in ASD (52). The duplication on 11q22.3 is classified as **VOUS; likely pathogenic**. The duplication on Xq21.31 is classified as **VOUS**.

Patient EMC-12 was a 23-year old male who had a diagnosis of PDD-NOS, ADHD, ODD, and substance abuse. It was estimated he had mild intellectual disability, but formal IQ testing was not performed. He exhibited a high receding hairline, a region of hyperpigmentation on the left shoulder, an upturned nose, and a coarse philtrum. Microarray screening revealed a gain for the entire Y chromosome, which was supported by gains in de pseudoautosomal regions located on X and Y. Karyotyping confirmed a single additional Y chromosome (47,XYY). The relationship between 47,XYY syndrome and psychiatric disorders is controversial. Studies of patients with 47,XYY syndrome have identified an increased risk of ASD and ADHD, as well as a higher burden of externalizing symptoms (64). This variant is classified as **pathogenic**.

Patient EMC-14 was an 18-year old male with a diagnosis of PDD-NOS, ADHD, and was a convicted sex offender. He had speech and language problems requiring special education. His IQ could not be determined and was it was estimated he had mild intellectually disability with VIQ lower than PIQ, while PIQ was average. He exhibited broad and prominent eyebrows with synophrys, long palpebral fissures, full pouching lips, a high and flat philtrum, obesity, tapering fingers, and hyperextension of the proximal interphalangeal joint. His height was 2.5 standard deviations (SD) below the mean. Genotyping revealed a 643 Kb deletion on the short

arm of chromosome 16 (16p11.2) overlapping with the critical region of the 16p11.2 microdeletion syndrome (61; 65; 66). This variant is classified as **pathogenic**.

Patient EMC-16 was a 43-year old male with a diagnosis of psychotic disorder NOS and had mild intellectual disability with an estimated total IQ of 75-85. He was lues-seropositive and treated with penicillin, but without classical signs of neurosyphylis nor any significant change in his Axis I symptoms following antibiotic treatment. He had a flat face, attached earlobes, hairy ears and prominent eyebrows. In addition, he had thoracic kyphosis and hyperpigmentation. Genotyping identified two CNVs: a 602 Kb duplication on the short arm of chromosome 3 (3p26.3) containing *CNTN6*, and a 595 Kb duplication on the short arm of chromosome 11 (11p11.12) containing pseudogenes *LOC441601* and *LOC646813*. In addition, several large regions of homozygosity were identified, consistent with the reported consanguinity of the parents who were not available for examination. *CNTN6* encodes the protein contactin 6, a cell adhesion molecule that functions to regulate central nervous system development. Mutations of *CNTN6* have been associated with inherited forms of cognitive and psychiatric disorders (67). In addition, mutations in this gene have been firmly associated with autism spectrum disorders (68; 69). The region of homozygosity did not contain genes that could be connected to the phenotype. The duplication on 3p26.3 is classified as a **VOUS; likely pathogenic**. The duplication on 11p11.12 is classified as a **VOUS**.

Patient EMC-31 was a 36-year old female with a diagnosis of psychotic disorder NOS and depressive disorder. It was estimated that the patient had mild intellectual disability, but formal IQ testing was not performed. Her social emotional development age was estimated to be 6 years. She had a compound heterozygous 66 Kb deletion and 230 Kb duplication on the short arm of chromosome 3 (3p26.3). In addition, she had a 717 Kb deletion on the long arm of chromosome 8 (8q21.13). Both of the chromosome 3 CNVs involve *CNTN4*. Mutations in *CNTN4* are associated with ASD intellectual disability, and growth retardation (70). In addition, the *CNTN4* locus was identified to among the genome-wide significant loci (P = 2.692 x 10^{-11}) in the most recent GWAS of SCZ by the Psychiatric Genetics Consortium (1). The deletion on chromosome 8 contains *CHMP4C* and *SNX16*. Fragile X testing was negative. The compound heterozygous variants on 3p26.3 are classified as **VOUS; likely pathogenic**. The deletion on 8q21.13 is classified as a **VOUS**.

Table 5. Characteristics for patients with positive genetic findings Erasmus MC

Pat nr.	EMC-1	EMC-4	EMC-9	EMC-10	EMC-11	EMC-12	EMC-14	EMC-16	EMC-21	EMC-30	EMC-31
Gender	Male	Male	Male	Male	Male	Male	Male	Male	Male	Female	Female
Age	34	37	53	22	27	23	18	43	55	50	36
Psychiatric disorder	Bipolar II disorder	PDD-NOS, psychotic disorder NOS, PTSD	Dysthymic disorder	PDD-NOS, intermittent explosive disorder	PDD-NOS, dysthyic disorder	PDD-NOS, ADHD, ADD, substance abuse	PDD-NOS, ADHD	Psychotic disorder NOS	Autistic disorder, depressive disorder NOS, OCD	Psychotic disorder NOS	Psychotic disorder, depressive disorder
FSIQ	76	69	55	69	85	Na	Na	Estimated 75-85	82	64	Estimated ID
PIQ		72	50	79	86					69	
VIQ		66	56	66						65	
Educational level	Lower-level secondary education	Special education	Special education	Special education	Special education	Special education	Special education	Primary education only	Lower professional education	Special education	Special education
Family history	Mother with bipolar disorder	Mother and siblings have learning disabilities	Alcohol abuse father	ID in sibling	No postive family history	Bipolar disorder and autism	Brother has ADHD	None, parents are consanguineous	Father has schizophrenia, sibling has psychotic disorder, son of stepmother has psychotic disorder, daughter of stepmother has ID	1 brother with schizophrenia and 1 brother with ID	1 brother with ID
Somatic symptoms	Diabetes, Hypothyroidism, hypercholesterolemia, cryptorchidism, amblyopia, retractile testes	None	Diabetes, hard of hearing (AD>AS), varices	Ear infections, back problems	None	Inguinal hernia requiring surgery	CARA, migraine, speech and language disorder	Hypermetropia, lues	Obesitas	None	None

Pat nr.	EMC-1	EMC-4	EMC-9	EMC-10	EMC-11	EMC-12	EMC-14	EMC-16	EMC-21	EMC-30	EMC-31
Facial charac.	Upslanted palpebral fissures, epicanthus, downturned corners mond, asymetric prominent jaw, carious dents, flat philtrum, high skull	Geheimratsecken, hypertelorism, broad palpebral fissures, brushy eyebrows, synophrys, prominent nose tip, narrow upper lip, edentate upper jaw,dysplastic ear helices	Elongated face, flattened center face, ptosis, narrow palpebral fissures, prominent pear-shaped nose	Hypoplastic alae nasi	Geheimratsecken	High hair line with geheimratsecken, turned-up nose tip, rough philtrum	Broad and prominent eye brows with synophrys, broad palpebral fissueres, full pouching lips, high and flat philtrum	Flattened face, attached earlobes, hairy ears, prominent eyebrows	Obesity, small palpabral fissures, hypoplastic aslae nasi, high papalate, long ears	Long face, pioma on the head, exophthalmus, full nose tip, hypotonic in the face	None
Body charac.	Edema on the lower legs, panniculus	Increased lordosis and kyphosis, mild pectus excavatum, ugly scar formation, striae on the upper legs, Beighton score 2/9, normal tonus, slim posture, hypotrophic muscular system	Length 1 SD below the mean, weight 2 SD above the mean, skull circumference 1 SD above the mean. central adipositas	None	Clinodactylia 5th finger, haemangioma LS, striae arms en abdomen	Hyperpigmentation on left shoulder	Length 2.5 SDs below the mean, adipous, tapering fingers, and hyperextension of the proximal interphalangeal joint	Increased thoracic kyphosis, hyperpigmentation, axial hypotonic, length 2.5 SDs below the mean, weight 2.5 SDs above the mean	Weight 2 SDs above the mean, hernia umbilicalis, panniculus, thoracal kyphosis, and long hands and feet	Length and skull circumference 0.5 SD below the mean, hallux valgus right>left, increase thoracic kyphosis	None
Microarray results	del5p13.2 (238kb)	dup7p22.3 (481kb)	del3q13.2q13.31 (3377kb)	del1q21.1 (1332kb)	dup 11q22.3 (336kb) and dupXq21.31 (217KB)	47, XYY	del16p11.2 (643 kb)	dup3p26.3 (602kb), dup11p11.12 (595kb)	dup5q21.1 (236kb)	del13q33.3 (1.7 Mb)	del3p26.3 (66 kb) & dup3p26.3 (230 kb), del8q21.13 (717 kb)

Pat nr.	EMC-1	EMC-4	EMC-9	EMC-10	EMC-11	EMC-12	EMC-14	EMC-16	EMC-21	EMC-30	EMC-31
Diagn. class	VOUS	VOUS	Pathogenic	Pathogenic	VOUS; likely pathogenic	Pathogenic	Pathogenic	VOUS; likely pathogenic	VOUS	VOUS	VOUS; likely pathogenic
ROH	None	None	None	None	None	None	None	Multiple, parents consanguineous	None	None	None
Metabolic screen blood plasma	Normal	Normal	Normal	Normal	Normal	Normal	Normal	High alanine and glutamine	Normal	Na	Na
Metabolic screen urine	Normal	Normal	Slightly elevated lactate levels	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Fragile X testing	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

FSIQ Full-scale IQ; PIQ performance IQ; VIQ verbal IQ; ID Intellectual disability; ROH Regions of homozygosity

DISCUSSION

We performed comprehensive genetic analyses in 2 cohorts of patients with a syndromic presentation of psychiatric disorders. In 12 (24%), we identified a pathogenic or likely pathogenic genetic variant. We found a similar percentage of pathogenic or likely pathogenic CNVs in each independent cohort (Pilgrim Psychiatric Center 5/19, 26.3%; Erasmus MC 7/31 22.6%). Among the identified CNVs, many have been previously established as known genetic risk factors for psychiatric disorders (22q11.2 microdeletion, 16p11.2 microdeletion, 47 XYY syndrome) and developmental delay (3q13.31 microdeletion, 1q21.1 microdeletion). Furthermore, we observed CNVs involving specific genes strongly linked to psychiatric disorders (*CNTN4, CNTN6)*. These CNVs have a broad range of penetrance (the chance that one will be affected given that one is carrier of the CNV). For risk assessment based on carriership, the penetrance data is very valuable. Nevertheless the conferred risk is considerable and merits to be taken into account for diagnosis and illness management (71–73).

The results from our assessment of two independent patient cohorts are highly consistent with a recent study by Stobbe and colleagues who reported clinical diagnostic findings of 24 consecutively evaluated adult patients with syndromic ASD of whom 20.8% were found to have pathogenic or likely pathogenic CNVs (74). The authors found that patients with syndromic forms of psychiatric disorders have a notably elevated frequency (20.0-22.6%) of pathogenic or likely pathogenic CNVs compared to patients with non-syndromic forms of psychiatric illness. In particular, CNVs currently established as pathogenic for psychiatric illness were markedly elevated in our syndromic cohort (7/50, 14%) compared to the general population SCZ cohort reported by Rees et al (5) (170/6882, 2.48%). Therefore, clinical genetic testing for CNVs may be particularly relevant in patients with syndromic forms of Axis I psychiatric disorders.

Genetic testing can be considered in the presence or absence of a significant family history. Whereas a positive family history suggests the presence of an inherited genetic variant, a negative family history can be indicative of a *de novo* variant. However, the absence of an identifiable inherited or *de novo* genetic variant does not rule out more complex genetic events due to somatic mosaicism that might not be detectable in DNA isolated form peripheral blood. The considerable diagnostic importance of *de novo* mutations has been firmly established for intellectual disability (16; 18; 75). Accordingly, this might also be an important genetic

mechanism underlying syndromic forms of psychiatric illness that should be evaluated in future studies, in particular for those variants classified as VOUS. Importantly, future investigations with larger cohorts should be performed to determine the relative contribution of intellectual disability versus distinct congenital abnormalities, facial dysmorphologies, and seizure disorder to the prior probability of CNVs in patients with psychiatric disorders.

In summary, we suggest that CNV screening should be considered for implementation within routine clinical practice for patients with syndromic forms of psychiatric illness. Although formal cost effectiveness analyses await completion, the current price of comparative genomic hybridization microarray testing is approximately $200 (76) with a number needed to test of 4.35 based on the combination of our results and those of Stobbe et al (17/74 with pathogenic or likely pathogenic variants). Moreover, with the exponentially increasing amount of available genetic data, an increasing proportion of the variants now considered VOUS on the basis of insufficient information are likely to be reclassified in the near future with even further benefits to the diagnostic yield of CNV testing.

Clinical Guidance

Pathogenic or likely pathogenic CNVs are present in a substantial fraction of patients with syndromic forms of psychiatric illness. Currently, the diagnostic yield appears to be the highest when genetic testing is implemented in patients with at least axis I psychiatric disorders and mild-to-moderate intellectual disability in combination with multiple congenital abnormalities and/or a dysmorphic features. Given that genetic variants are often *de novo*, genetic testing should still be considered even if there is a negative family history for the syndromic psychiatric and medical features observed in the proband.

Clinical Vignette

Mr. A is 22-year-old male patient with a slim appearance who lives at home and helps out in his parents' convenience store. During the last 2 years, he has been in a romantic relationship with a woman from the same town where he lives. Mr. A experienced his first psychotic episode at the age of 17, during which he was hospitalized for a duration of 6 weeks and treated with 5 mg of haloperidol which led to a significant reduction in his psychotic symptoms. Mr. A was discharged with a diagnosis of psychotic disorder NOS and began outpatient visits with a psychiatrist. During the first year following discharge from the hospital, his medication regimen was transitioned to 4 mg of risperidone due to extrapyramidal

side effects on haloperidol and his diagnosis was revised to schizophrenia. Over the past 5 years, Mr. A has experienced several periods of increased psychotic symptoms. However, these were well controlled by temporarily increasing the dose of risperidone. During subsequent family meeting with Mr. A and his parents, it became apparent that Mr. A's mother suffers from intermittent non-affective psychotic episodes, which had never been formally evaluated or treated. The family agreed that Mr. A bears a close resemblance to his mother, both in terms of his psychiatric symptoms as well as his physical appearance. The mother also reported having a younger sister who she described as "a little odd". Throughout elementary school, Mr. A performed below average and was late in reaching his developmental milestones. His elementary school teacher had suggested to his parents that he might suffer from ADHD, but this was never formally evaluated. In secondary school, Mr. A continued to struggle academically and decided to stop attending school beyond the legal requirement at age 16. During the course of his outpatient treatment, neuropsychological testing revealed that Mr. A had substantial impairments in both attention and working memory. His FSIQ was determined to be 70 (VIQ 68, PIQ 72). Coincidentally, the family read an article in their local newspaper about genetic testing in patients with autism spectrum disorder, which prompted Mr. A to discuss genetic testing with his psychiatrist who referred him for clinical genetic counseling. During the physical examination by the clinical geneticist, Mr. A was noted to have dysmorphic facial features, including elongated face with low-set ears, elongated fingers and toes, and a flat nasal bridge. Oral examination revealed a high-arched palate. Genotyping of Mr. A and his parents revealed a maternally-inherited 16p11.2 duplication. Brain MRI was notable for cavum septum pellucidum and enlargement of the 3rd and 4th ventricles.

The psychiatric symptoms and dysmorphic features of Mr. A and his mother are consistent with the 16p11.2 microduplication syndrome (OMIM #614671). The genetic counselor discussed the findings and the implications for future offspring with Mr. A and his parents. Both Mr. A and his parents reported that they were comforted to learn that there was an identifiable cause of the psychiatric illness of Mr. A and his mother. However, the patient's mother also described experiencing some ambivalence about the outcome of the genetic testing, with feelings of guilt about her responsibility in "passing on" the pathogenic CNV to her son. The mother's concerns were discussed over several sessions with a clinical psychologist who is an integral member of the medical genetic counseling team. Mr. A is now aware that there is a 50% chance for each of his children to inherit the 16p11.2 microduplication. Mr. A and his partner have expressed a desire to have children and were

specifically counseled about family planning. Additional family members potentially at risk for carrying the 16p11.2 microduplication by Mendelian inheritance were provided the contact information of the clinical geneticist by Mr. A and his mother about the option for them to consider a genetic evaluation for the risk variant.

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SUPPLEMENTARY INFORMATION

Cases with only variants classified as VOUS

Pilgrim Psychiatric Center

Patient PPC-2 was a 32-year-old male diagnosed with schizophrenia, disorganized type with a chronic course. The first episode of psychosis occurred at age 15. The patient completed an 11th grade education. WAIS-III-R testing revealed a VIQ of 75, PIQ of 79, and FSIQ of 75. The patient was noted to have an elongated face and pectus excavatum. Medical history was only notable for psychogenic polydipsia. MRI revealed fronto-parietal white matter foci; cerebral and cerebellar atrophy; cavum septum pellucidum; asymmetric prominence of the lateral ventricles; pointing and prominence of the 4th ventricle. Palatal assessment revealed a submucous cleft palate. We identified a 343 Kb duplication on the long arm of chromosome 22 (22q13.22), containing *FLJ44385* and *C22orf34*. These genes are not known to be associated to the phenotype.

Patient PPC-11 was a 40-year-old male who had his first psychotic episode at age 18 and subsequently received a diagnosis of undifferentiated schizophrenia with a chronic course of illness. The patient was also diagnosed with intellectual disability as a child and attended special education classes. WAIS-III-R testing revealed a VIQ of 68, PIQ of 65, and FSIQ of 64. Dysmorphic features include a sloping forehead; protruding supra-orbital ridges; square nasal root with a bulbar nose and wide nasal tip; short philtrum; retrognathia; malar flatness and a short and broad sternum. Palatal assessment revealed a submucous cleft palate. We identified a 2.11 Mb duplication on the short arm of chromosome 16 (16p12.3) containing *NPIPA7, NPIPA8, PKD1P6-NPIPP1, NOMO2, RPS15A, ARL6IP1* (associated with autosomal recessive spastic paraplegia (1), *SMG, TMC7, COQ7* (associated with autosomal recessive coenzyme Q10 deficiency (2)), *ITPRIPL2, SYT17, TMC5, GDC1, CCP110, C16ORF62, IQCK, GPRC5B,* and *GRP139*.

Patient PPC-15 was a 42-year-old female with a diagnosis of undifferentiated schizophrenia with a chronic course and with prolonged hospitalization (>20 years). WAIS-III-R testing revealed a VIQ of 61, PIQ of 69, and FSIQ of 61. The patient only completed a 9th grade education and was never able to work due to her mental illness. The patient was noted to have a high nasal root protrusion and kyphosis. Medical history is notable for psychogenic

polydipsia with hyponatremia and a history of seizures secondary to hyponatremia. MRI revealed asymmetric prominence of lateral ventricles; prominent left Sylvian fissure; prominence of cerebrospinal fluid (CSF) space adjacent to superior parietal lobule. Palatal assessment did not reveal any abnormalities. We identified a 237 Kb duplication on the short arm of chromosome 4 (4p16.3-16.2) containing *STX18* responsible for intracellular transport (3) and *STX18-AS*.

Patient PPC-16 was a 48-year-old male diagnosed with chronic paranoid schizophrenia with a chronic, unremitting course. The patient's first episode of psychosis occurred at the age of 18. The patient graduated high school, after which he joined the U.S. Navy but was discharged 9 months after enrollment due to a first-episode psychosis. The patient reportedly had normal developmental milestones and his medical history is notable for mitral valve prolapse with mitral regurgitation, status post repair and seizures that have occurred exclusively in the context of treatment with clozapine. The patient is noted to have narrow orbital fissures, retrognathia, protruding supra-orbital ridges and pectus excavatum. Neuropsychological testing was not done since the patient was not willing to participate. Palatal assessment was not performed. We identified a 499 Kb duplication on the long arm of chromosome 6 (6q26) containing *PARKIN* which is associated with autosomal recessive early-onset Parkinson's disease (4) and *PACRG* which is the PARKIN-coregulated gene (5).

Patient PPC-18 was a 36-year-old male with a diagnosis of schizoaffective disorder, characterized by a chronic course with continued loss of functional capacity. The patient was first evaluated by a psychiatrist at age 14 for psychotic symptoms and was first hospitalized at age 18. The patient graduated high school and completed 6 months of college after which he dropped out. The patient never worked and has been living in a residential treatment facility. WAIS-III-R testing revealed a VIQ of 92, PIQ of 83, and FSIQ of 90. Medical comorbidity was notable for hypothyroidism, psychogenic polydipsia with hyponatremia, chronic neutropenia, hiatal hernia and obesity. In terms of dysmorphisms, the patient was noted to have a high/protruded nasal root and with flat nasal alae; diminished vermillion of the upper lip, clefted nasal tip as well as mild midline cleft of lower lip; hypoplastic teeth (upper and lower jaw), hyoplastic earlobes, slender, tapered ('carrot-like') fingers. Palatal assessment revealed a high-arched, V-shaped, steepled submucous cleft palate. We identified a 510 Kb

duplication on the short arm of chromosome 2 (2p21) containing *SRBD1*. This gene is not known to be associated with the phenotype.

Erasmus University Medical Center

Patient EMC-1 was a 34-year-old male diagnosed with bipolar II disorder and substance abuse. With a total IQ of 76 he classified as having mild intellectual disability. Dysmorphological and physical examination of our patient indicated a high forehead, upslanted palpebral fissures, epicanthic fold, a flat philtrum, downturned corners of the mouth, carious dentition and an asymmetrical and prominent jaw. Furthermore, he had edema of the lower legs and central obesity. The patient had a 238 Kb deletion on the short arm of chromosome 5 (5p13.3), harboring the majority of *WDR70*. The clinical implication of this variant is uncertain. Five patients have been previously described with 5p13 duplication syndrome which included developmental delay, intellectual disability, congenital abnormalities and facial dysmorphology, however in only one case was *WDR70* in the duplication region (OMIM #613174).

Patient EMC-4 was a 37-year-old male who suffered from PDD-NOS, PTSD, and psychotic disorder NOS. He had mild intellectual disability with a total IQ of 69. Our patient had hypertelorism, broad palpebral fissures, brushy eyebrows, synophrys, a prominent nose tip, narrow upper lip, an edentate upper jaw, and dysplastic helices of the ear. Furthermore, he had increased lordosis and kyphosis, mild pectus excavatum, aberrant scar formation, striae on the upper legs, no hypermobility (i.e., a Beighton Hypermobility Score of 2/9), a slim posture, and a hypotrophic muscular system, with normal body length and skull circumference. Genotyping relevaled a 481 Kb duplication on the short arm of chromosome 7 (7p22.3), containing *FAM20C*. No patients with this duplication are described in the structural variant databases with this duplication. Autosomal recessive loss-of-function mutations of *FAM20C* in this gene cause Raine Syndrome, a rare autosomal recessive form of osteosclerotic bone dysplasia (OMIM #259775). The functional effect of *FAM20C* duplication is unclear. Agiropoulos and colleagues describe a patient with intellectual disability, short stature, microcephaly, and dysmorphic features that was found to have both a chromosome 7 *de novo* terminal deletion and a chromosome 7 terminal duplication which included *FAM20C* (6).

Patient EMC-21 was a 43-year-old male with a diagnosis of autistic disorder, depressive disorder, and OCD. The patient had mild intellectual disability. Total IQ was determined to be 82. He was obese, had narrow palpebral fissures, hypoplastic alae nasi, a high palate, long ears, hernia umbilicalis, panniculus, thoracic kyphosis, and had long hands and feet. Body weight was 2 SDs above the mean. We identified a 236 Kb duplication on the long arm of chromosome 5 (5q21.1) containing *FAM174A*. *FAM174A* is abundantly expressed in all organ systems, including the brain.

Patient EMC-30 was a 50-year-old female with a diagnosis of brief psychotic disorder and mild intellectual disability with a total IQ of 64. Our patient had a long hypotonic face, exophthalmos, a full nose tip, a hallux valgus (right>left), and thoracic kyphosis. Genotyping revealed a 1.7Mb deletion on the long arm of chromosome 13 (13q33.3) containing *FAM155A, LIG4, ABHD13, and TNFSF13B*. No patients with a similar loss have been described in the DECIPHER database. Homozygous mutations in *LIG4* are associated with *LIG4* syndrome/Nijmegen breakage syndrome that includes facial dysmorphisms, developmental delay, and immunodeficiency (7).

Supplementary references

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Part IV |

Discussion

8

De psychodynamiek van een belaste familiegeschiedenis

Christian G. Bouwkamp, Mijke P. Lambregtse - van den Berg, Anneke J.A. Kievit, Steven A. Kushner

Auteurs

Christian G. Bouwkamp, psycholoog & promovendus, afdeling Psychiatrie en afdeling Klinische Genetica, Erasmus MC Rotterdam

Mijke P. Lambregtse – van den Berg, (kinder- en jeugd) psychiater & psychoanalytisch psychotherapeut, afdeling Psychiatrie en Kinder- en Jeugdpsychiatrie/Psychologie, Erasmus MC Rotterdam

Anneke J.A. Kievit, klinisch geneticus & opleider klinische genetica, afdeling Klinische Genetica, Erasmus MC

Steven A. Kushner, psychiater & hoogleraar Neurobiologische Psychiatrie, afdeling Psychiatrie, Erasmus MC

Manuscript in voorbereiding

Samenvatting:

Achtergrond: Een positieve familiegeschiedenis is de belangrijkste voorspeller voor het ontwikkelen van psychopathologie. Vaak wordt het psychologisch effect van een positieve familiegeschiedenis niet voldoende onderkend, zowel in de klinische als niet-klinische populatie.

Doel: Inzichtelijk maken wat de onderliggende psychodynamiek is van kinderen uit gezinnen met een sterke belasting voor psychose, door te onderzoeken hoe deze familiaire belasting interfereert met de vroege ontwikkeling en in het latere leven. We onderzoeken verder hoe de effecten van deze interferentie mogelijk voorkomen kunnen worden.

Methode: Literatuuronderzoek, bespreking van theoretische concepten en aanbevelingen.

Resultaten en conclusie: Een positieve familiegeschiedenis kan de normale ontwikkeling van identiteit en het zelfgevoel ernstig verstoren. Interventies in de breedste zin van het woord kunnen deze effecten verminderen.

Summary:

Background: A positive family history is the most important predictor of the risk to develop psychopathology. Often, the psychological effect of a positive family history is not sufficiently acknowledged, both in the clinical as well as the non-clinical population.

Aim: To provide insight into the psychodynamics of children from families with psychosis in the family, how these effects can last a lifetime, and how this might be prevented.

Method: Selected literature review, discussion of theoretical concepts, and recommendations.

Results and conclusion: A positive family history has the potential to seriously disrupt normal development. Interventions in the broadest sense of the word can reduce this potential.

Introductie

Een positieve familiegeschiedenis voor psychiatrische stoornissen is de belangrijkste voorspeller voor het optreden van psychiatrische klachten bij asymptomatische familieleden. Hoe nauwer iemand genetisch verwant is met het aangedane familielid, hoe groter de kans op dezelfde of een daaraan gerelateerde aandoening (Austin and Peay, 2006). Recent grootschalig epidemiologisch onderzoek toonde al aan dat bij de etiologie van deze stoornissen een sterke erfelijke component betrokken is (Polderman *et al.*, 2015; Sullivan *et al.*, 2012), maar in de meeste gevallen is de precieze moleculair-genetische oorzaak onbekend. Ook wij bestuderen de genetische basis van familiaire vormen van psychiatrische stoornissen, omdat we geïnteresseerd zijn in de veranderingen in het erfelijk materiaal die de kans op het ontwikkelen van psychiatrische stoornissen sterk vergroten. Dit kan op termijn leiden tot inzicht in de pathofysiologie van deze stoornissen en een diagnostische waarde hebben. Psychiatrische stoornissen zijn ingrijpend voor de patiënt en de familie en ze hebben impact op veel aspecten van het leven. Wanneer iemand uit een familie komt waarin meerdere familieleden in meerdere generaties ernstige psychische klachten hebben gaat dit impliciet of expliciet gepaard met een zware emotionele belasting, met angsten en verlieservaringen. Gezien de erfelijkheid van psychiatrische aandoeningen zullen veel zorgverleners met patiënten te maken hebben die een positieve familieanamnese hebben, maar de familiegeschiedenis speelt vaak geen expliciete rol in diagnostiek en behandeling.

Het doel van dit essay is de onderliggende psychodynamiek te beschrijven van het afkomstig zijn uit een gezin waarin psychiatrische stoornissen voorkomen, met een focus op psychotische stoornissen. We beschrijven aan de hand van een aantal psychodynamische theorieën hoe dit bijdraagt aan de identiteit en het zelfgevoel. Hiervoor gebruiken we een selectie uit de bestaande literatuur.

Vignet van een sterk belaste familie

Wij zagen een familie waarin vier van de negen broers en zussen schizofrenie hadden. De oudste dochter, die we Maria zullen noemen, nam het voortouw in het contact. Maria had vanaf de lagere school de taak op zich genomen te helpen in het gezin waarin vader psychiatrische klachten ontwikkelde die ook opnamen vereisten in een psychiatrisch ziekenhuis. Dit viel in de tijd samen met de luxatie van psychosen van vier broers en zussen van Maria. Zij was het aanspreekpunt geworden voor alle administratieve en medische zaken van het gehele gezin. Wij zagen de familie in hun ouderlijk huis; in een flat waarvan er veel zijn

gebouwd na de Tweede Wereldoorlog. Maria was een vriendelijke kalme vrouw van middelbare leeftijd. Het familieverhaal was als volgt: naast hun vader ontwikkelden vier van de negen broers en zussen psychosen, met later de diagnose schizofrenie. Ook eerdere generaties van de familie werden getekend door ernstige psychiatrische problematiek en opnamen in psychiatrische ziekenhuizen. Reeds als kind hielp Maria moeder waar nodig, werd zij belast met de zorgtaken en ging niet naar de middelbare school. In plaats daarvan weidde zij zich geheel aan de zorg voor haar broers, zussen en vader. Een lange geschiedenis volgde van contacten met de regionale GGZ instelling, er volgden vele opnames van haar broers en zussen in verschillende psychiatrische klinieken. Ook was er veelvuldig contact met psychiaters, artsen, psychologen en sociaal psychiatrisch verpleegkundigen. Te midden hiervan zorgde Maria decennialang dat de doktersafspraken werden nagekomen en dat iedereen in het gezin zijn of haar voorgeschreven medicatie innam. Ook reguleerde ze het gebruik van alcohol en verbood het gebruik van drugs. De aangedane familieleden hadden ons inziens een opmerkelijk goede kwaliteit van leven in vergelijking met bijvoorbeeld gehospitaliseerde mensen met een vergelijkbare ernst van psychiatrische klachten. De meeste gezinsleden woonden in de buurt van het ouderlijk huis en twee van de broers en zussen woonden bij hun ouders. Deze langdurige mantelzorg van Maria voor haar broers, zussen en ouders heeft een diepe en blijvende impact gehad op haar identiteit. Zij is nu in de vijftig en beide ouders zijn inmiddels overleden. Maria noch haar broers en zussen hebben kinderen gekregen. Dit was het resultaat van de genetische analyse van de familie zelf: zij waren van mening dat zoveel mensen ernstige psychiatrische klachten hadden ontwikkeld dat het ze geen goed idee leek zelf aan nageslacht te beginnen. Na het overlijden van beide ouders is er het een en ander veranderd in het gezin: twee van de aangedane broers en zussen zijn op zichzelf gaan wonen met begeleiding en twee van de broers en zussen zijn niet meer in leven ten gevolge van suïcide. Nu, als vrouw van middelbare leeftijd, zonder schoolopleiding en zonder formele werkervaring dringt zich een leegte op bij Maria. Na jaren van intensieve zorgtaken is er ineens tijd en ruimte gekomen om eigen interesses en bezigheden te ontplooien. Voorzichtig onderzoekt zij hiervoor de mogelijkheden.

In dit essay onderzoeken wij het effect van psychopathologie bij de ouders op de identiteit van het kind en de ontwikkeling van het zelfgevoel. Wanneer het voor de aangedane ouder niet duidelijk is wat realiteit is, kan dit overgedragen worden via de ouder-kind relatie en dit kan de ontwikkeling van de identiteit en het zelfgevoel ernstig verstoren.

Psychiatrische klachten in het gezin

Hechting en de ouder-kind relatie

In de vroege ontwikkeling van een kind is de emotionele en fysieke beschikbaarheid van de ouders c.q. primaire verzorgers cruciaal (wij richten ons hier primair op de ouder-kind relatie van biologische ouders met psychopathologie, aangezien de insteek is om naar de psychodynamiek te kijken in de context van een belaste familiegeschiedenis), aangezien zeer jonge kinderen in het vervullen van hun primaire levensbehoeftes nog volledig afhankelijk zijn van volwassenen. Hiervoor is het essentieel dat de lichamelijke en emotionele signalen van een baby voldoende herkend en beantwoord worden door de ouders. In de ontwikkeling vormt het kind via de ouder een intern werkmodel en bouwt interne representaties op over hoe de ander zich tot hem/haar verhoudt in het bieden van veiligheid en geborgenheid. Het onvoldoende kunnen bieden van een veilige omgeving c.q. *holding environment* leidt tot een onveilige gehechtheid. Een onveilige gehechtheid maakt kinderen kwetsbaar in het omgaan met interpersoonlijke stress. De aanwezigheid van sterke psychiatrische problematiek in de familie kan de ontwikkeling ernstig verstoren en psychopathologie bij de primaire hechtingsfiguur in het gezin kan de mate waarin een veilige *holding environment* wordt geboden sterk verminderen (Schechter and Willheim, 2009). Het jonge kind is volledig afhankelijk van de ouders voor de primaire levensbehoeften en het ontwikkelen van een fundament voor interpersoonlijke contacten. Dit is een precair proces waarbij het belangrijk is dat de ouders en het kind op elkaar afgestemd zijn. Wanneer er bij de ouder sprake is van psychiatrische klachten, en in het bijzonder psychotische klachten, wordt deze afstemming sterk bemoeilijkt. Daarom dient er in de behandeling in het geval van psychose voldoende aandacht te zijn voor de kwaliteit van de ouder (moeder)-kind diade. In bepaalde gevallen, zoals in het geval van postpartum psychose, kan het zelfs een reëel gevaar opleveren voor het kind aangezien deze onderdeel kan zijn van de psychotische belevingen. In dit geval dient de ouder onmiddellijk te worden behandeld volgens de richtlijn (Bergink *et al.*, 2015).

Effect van psychopathologie op het zelfgevoel en identiteit

De hechtingstheorie van de Engelse psychoanalyticus John Bowlby is gerelateerd aan de ontwikkeling in relatie tot de ouder. Hij stelde dat de vroege persoonlijkheidsontwikkeling plaatsvond in de diade met de ouder (meestal de moeder) (Bowlby, 1952). Onderzoek wees uit dat het kind er alles aan doet om contact te behouden of te krijgen met de primaire hechtingsfiguur. Wanneer de ouder bijvoorbeeld onvoldoende in staat is een veilig

opvoedingsklimaat te bieden, bijvoorbeeld door de aanwezigheid van psychotische klachten, kan de ontwikkeling van identiteit en zelfgevoel sterk ontregeld raken. Dit fenomeen is goed te observeren in de *strange situation test* ontwikkeld door Mary Ainsworth, waarbij wordt gekeken hoe het kind reageert op het plotselinge vertrek van de ouder in het bijzijn van een vreemde, op geruststellende interactie met de vreemde en op de hereniging met de ouder (Ainsworth and Bell, 1970). Ook het *still face* experiment waarbij de ouder na normale interactie plotseling een pokerface opzet, heeft als effect dat het kind probeert met escalerende gedragingen contact te krijgen met de ouder (Tronick and Gianino, 1986). Beide laten zien dat er een sterke wisselwerking is tussen de ouder/verzorger en het kind. Ouders met psychiatrische stoornissen zijn verminderd emotioneel beschikbaar wat zich uit in een verminderde affectspiegeling. Kinderen van ouders die chronisch verminderd emotioneel beschikbaar zijn gaan zich terugtrekken (overregulatie) of blijven voortduren een angstig appèl doen op de ouder (onderregulatie). Kinderen uit een gezonde diade kunnen zich echter goed herstellen van een tijdelijke verstoring. Vervolgonderzoek liet zien dat hechtingsstijlen een blijvend effect hebben op de kwaliteit van relaties op latere leeftijd (Lieberman *et al.*, 1999).

Psychiatrische klachten gaan vaak gepaard met gevoelens van verlies en vervreemding. Vaak zijn deze gevoelens anaclytisch van aard en gaat het om een verliesgevoel in relatie tot de ander. Door het effect van de psychiatrische problematiek op de ander neemt deze op zijn beurt afstand van de patiënt. De patiënt ervaart dit op zijn beurt weer als een afwijzing van de ander. Bij schizofrenie of bipolaire stoornis, die kunnen ontstaan na een lange periode van normaal functioneren, moeten er aanpassingen gedaan worden aan de verwachting van het (dagelijks) leven. Het kan zijn dat de eisen die een baan stelt te hoog zijn geworden of dat een werkgever het niet tolereert dat iemand langere tijd uit de roulatie is en het contract niet verlengt. Het kan ook zijn dat vrienden en bekenden afgeschrikt worden door het confronterende aspect van psychiatrische stoornissen, zoals het op bezoek gaan bij een vriend op een opnameafdeling. Daarnaast dient vaak dagelijks medicatie te worden genomen, die de nodige bijwerkingen kan hebben. Dit zijn verschillende verlieservaringen die iemand naast zijn psychiatrische klachten moet incasseren en dit hangt samen met het gevoel van het "*verlies van het gezonde zelf*" (Frank, 2005). Tot het moment dat de patiënt een nieuw evenwicht heeft gevonden waarbij de psychiatrische klachten geassimileerd zijn, zullen er perioden zijn dat hij of zij in strijd is met de diagnose. Er kan twijfel bestaan aan deze diagnose en aan de noodzaak van farmacotherapie: de patiënt zal zich afvragen of hij of zij niet zonder medicatie kan. Dit

laatste kan soms een redelijke vraag zijn, maar zal vaak samenhangen met een sterke verslechtering van de situatie wanneer er daadwerkelijk sprake is van een ernstige psychiatrische stoornis. Ondersteunende gesprekken, psycho-educatie en psychotherapie kunnen worden aangewend wanneer de acceptatie van het hebben van psychiatrische klachten en consequent medicatiegebruik moeilijk blijft (Oud *et al.*, 2016; Turner *et al.*, 2014).

Een ouder hebben met een psychotische stoornis

Chronische aandoeningen hebben een sterke invloed op de dynamiek van het gezin. Bekend is dat familieleden van patiënten met hersenaandoeningen vaker lijden aan angst- en stemmingsstoornissen (González-Salvador *et al.*, 1999; Lowery *et al.*, 2000). Recent verscheen er een overzichtsstudie uit Scandinavië waarin de literatuur wordt weergegeven van de subjectieve ervaringen van zogenaamde *KOPP* kinderen: kinderen van een ouder met psychiatrische problemen. Uit dit onderzoek bleek dat kinderen van ouders met psychiatrische problematiek erg zijn afgestemd op de aangedane ouder, dat zij de ouder in de gaten houden, taken overnemen waar dat nodig is en niet tot last willen zijn. Ook schamen zij zich voor de situatie en nemen waar mogelijk enige afstand tot de aangedane ouder (Dam and Hall, 2016). Kinderen van ouders met psychiatrische problematiek gaven in een Zweedse studie aan dat ze het als positief ervoeren wanneer een aangedane ouder was opgenomen omdat dit even lucht bracht in de thuissituatie. Als negatief ervoeren ze het gebrek aan informatie van het behandelteam aan hen als kinderen van aangedane ouders. De onderzoekers geven als advies om kinderen van ouders met psychiatrische problematiek adequaat te informeren over de klachten van hun ouders om zo de veerkracht te stimuleren en de psychopathologie van de ouder in een beter perspectief te zien (Knutsson-Medin *et al.*, 2007). In een andere Zweedse studie, met als doel de ervaringen van alledag van kinderen tussen de 10 en 18 jaar oud met een opgenomen ouder inzichtelijk te maken, werd gevonden dat deze kinderen ervoeren dat ze relatief geïsoleerd waren met hun gedachten over de aangedane ouder en dat ze het belangrijk vonden de situatie te kunnen bespreken met de gezonde ouder of met anderen. Kinderen benoemden ook hun angst, voornamelijk met betrekking tot het proces van ziek worden en een mogelijke suïcide bij de ouder. Daarnaast kwam naar voren dat de kinderen zich pijnlijk bewust waren van het feit dat de aangedane ouder zich onvoldoende in kon zetten voor een goede opvoeding. Kinderen hielden van hun aangedane ouder, maar het bleek van belang dat ze niet verstrikt raakten in de denkwereld van de aangedane ouder (Ostman, 2008). In dit kader is het aan te raden, wanneer er een kinderwens speelt bij een ouder met ernstige

psychiatrische klachten, al vroeg aan te vangen met interventies om het ouderschap in goede banen te leiden en transgenerationele overdracht van psychopathologie te vermijden (Lambregtse-van den Berg, 2015; Nijssens *et al.*, 2015). Wanneer het kind er eenmaal is zal het belangrijk zijn te monitoren dat de ontwikkeling van het kind in goede banen geleid wordt middels een multidisciplinaire aanpak waarbij de naaste zorgverleners van het kind en ouder betrokken zijn. Daarnaast is het van belang het mentaliseren te bevorderen zodat de ouder leert dat het kind een eigen binnenwereld heeft die niet samenvalt met die van de ouder. Wanneer dit onvoldoende is, is verwijzing naar de specialistische GGZ geïndiceerd (Vliegen and Ruijten, 2015).

Parentificatie

Wanneer de ouders niet voldoende beschikbaar zijn voor het kind vanwege een psychiatrische aandoening, kan er een rolomkering (parentificatie) plaatsvinden waarin het kind zich verantwoordelijk gaat voelen voor de ouder in plaats van omgekeerd. Parentificatie treedt op wanneer er onredelijke verwachtingen zijn ten opzichte van het kind in een gedesorganiseerde thuissituatie. Het kind moet een *holding environment* scheppen voor de ouder in plaats van andersom (Chase, 1999). Deze overvraging interfereert met de individuatie en autonomie van het kind. Er is aangetoond dat de effecten hiervan zich in de adolescentie en volwassenheid doorzetten (Earley and Cushway, 2002). Uit een studie met kinderen in de leeftijd tussen 8 en 17 jaar kwam naar voren dat voorspellers van parentificatie, wanneer de moeder is aangedaan, bestaan uit: afkomstig zijn uit een eenouder gezin, enig kind zijn en de aanwezigheid van een persoonlijkheidsstoornis bij de ouder. Er werd een negatief effect gevonden van een jongere leeftijd van het kind, een kind van het mannelijk geslacht en een lager opleidingsniveau van de moeder. Er is met name sprake van overvraging wanneer het aankomt op aannemen van emotionele zorgtaken. Het is normaal wanneer kinderen op enigerlei wijze meehelpen in het huishouden, maar de studie concludeert dat het schadelijk is als er te veel van het kind wordt gevraagd in een context die niet steunend, niet responsief en niet validerend is (McMahon and Luthar, 2007). Parentificatie is ook gebleken transgenerationeel overdraagbaar te zijn: moeders die zelf rolomkering hebben meegemaakt met hun eigen moeder hadden hier ook vaker mee te maken in de relatie met hun dochters. Wanneer vaders rolomkering hadden meegemaakt met hun moeder, bleek het vaker zo te zijn dat er ook sprake was van rolomkering in de relatie tussen zijn vrouw en zijn zoon (Macfie *et al.*, 2005).

Angst voor de eigen toekomst

Wanneer er familiaire aanleg is voor psychiatrische of somatische aandoeningen is het vaak zo dat asymptomatische familieleden angst hebben om ook ziek te worden. Gezien het verhoogde risico voor naaste verwanten van patiënten met bijvoorbeeld schizofrenie of bipolaire stoornis, is er een reële kans dat ook zij deze klachten ontwikkelen wanneer bijvoorbeeld één van de ouders, broers of zussen schizofrenie of een bipolaire stoornis heeft. Identificatie met de aangedane ouder kan een grote rol spelen, zoals ook het geval is bij de ziekte van Huntington (Tassicker, 2005). Sterk bepalend is daarbij het beeld dat iemand heeft van familieleden die symptomatisch zijn. Identificatie met deze personen kan optreden, zeker wanneer het naaste familie betreft zoals bijvoorbeeld een ouder. Het concept identificatie verwijst naar het proces dat iemands persoonlijkheid samengesteld is uit een mozaïek van geïnternaliseerde representaties van significante anderen. Aangezien de ouders de primaire hechtingsfiguren zijn, kan het deel psychopathologie bij de ouders ook geïnternaliseerd worden en eventueel leiden tot een gevoel van determinisme. Onderzoek bij mensen waarbij de ziekte van Huntington in de familie speelt wees uit dat op het moment dat iemand van de ene levensfase naar de andere overgaat (1) jong volwassene, 2) jong stel, 3) jong gezin, 4) gezin met kinderen in de adolescentie, 5) kinderen het huis uit en 6) gezin op latere leeftijd) er sprake is van een opleving van de onzekerheden ten aanzien van de erfelijke aandoening in de familie (Brouwer-Dudokdewit *et al.*, 2002). Dit betreft de uitkomsten van moleculair erfelijkheidsonderzoek, maar ook de wetenschap van een sterk verhoogde kans op het ontwikkelen van de ziekte van Huntington gegeven dat een van de ouders ook aangedaan was. De ontwikkeling van depressieve gevoelens kan sterker zijn in het geval van psychopathologie bij de ouders, die samenhangt met sterke verlieservaringen in het verleden (Vliegen and Ruijten, 2015). Het in acht nemen van eerdere ervaringen ten aanzien van objectrelaties kan waardevol zijn bij het onderzoeken hoe iemand omgaat met de verhoogde kans om ziek te worden en in het geval dat de eerste symptomen zich voordoen. Familietherapie met een focus op de objectrelaties kan eventueel uitkomst bieden deze verlieservaringen te verwerken en de overgangen van de levensfasen waarbij een opleving plaatsvindt van angst of onzekerheid te vergemakkelijken (Diekmann-Tapon, 1999).

Veerkracht bij psychiatrie in de familie

Ieder mens is in meer of mindere mate in staat om negatieve omstandigheden (acuut of chronisch) te incasseren en hier veerkrachtig op te reageren. Dit is relevant in de context van

het opgroeien bij een ouder met psychiatrische klachten. Er is veel onderzoek gedaan naar veerkracht, meestal aangeduid met de Engelse term *resilience*, bij kinderen die opgroeien in aversieve omstandigheden, zoals bijvoorbeeld in het geval van psychopathologie bij de ouders. Bleuler beschreef al dat kinderen van ouders met schizofrenie leerden differentiëren tussen het zieke en het gezonde deel in hen (Bleuler, 1978; Ostman, 2008). Onderzoek wees uit dat in veerkrachtige kinderen de competenties van de normale ontwikkeling ondanks aversieve omstandigheden gewaarborgd blijven en de ontwikkeling zodoende niet bedreigd wordt. De belangrijkste aspecten hierbij zijn een goed intellect en een goede relatie met gezonde volwassenen (Masten and Coatsworth, 1998).

In een studie in Zuid-Afrika werd gekeken naar de aspecten van veerkracht in families met schizofrenie en er werd gevonden dat er drie domeinen waren die samenhingen met veerkracht in deze situatie. Dit waren 1) sociale ondersteuning, 2) eigenschappen van de familie en 3) eigenschappen van het aangedane familielid (Bishop and Greeff, 2015). De onderzoekers lieten zien dat openheid naar andere familieleden, vrienden en de gemeenschap beter leek te zijn en dat de situatie een mogelijkheid biedt nieuwe kennis op te doen over psychiatrische problematiek. Verder kan een gezonde hechte familieband de situatie verlichten. Het bleek belangrijk om de aangedane familieleden zich volwaardig en waardevol te laten voelen waarbij ook hoorde dat ze hun voorgeschreven medicatie innamen en zich op de hoogte stelden van de eigenschappen van hun aandoening zodat ze daar steeds beter mee leerden omgaan. Tot slot bleek dat psychopathologie bij de ouders in de weg stond van een gezonde coping van het kind met de psychische klachten van de broers en zussen. Het strekt tot aanbeveling de kinderen van de patiënt op een adequate manier te betrekken bij de behandeling en te proberen mythevorming over waarom de ouder ziek is bij kinderen weg te nemen door transparant te zijn over de stoornissen van de ouder.

De rol van de medische genetica

Er wordt veel onderzoek gedaan naar de onderliggende genetische oorzaak en pathofysiologie van psychiatrische stoornissen. In Amerika is onderzoek gedaan naar de interesse in erfelijkheidsonderzoek van ouders van pasgeboren kinderen. Hypothetisch zou dan de mogelijkheid bestaan alle huidig bekende en toekomstige genetische risico's op ziekte in kaart te brengen (iets wat nog niet mogelijk is in de psychiatrie) (Waisbren *et al.*, 2016). De uitkomst was dat wanneer er sprake was van stress bij de ouders en wanneer er zorgelijke medische

informatie met betrekking tot het kind was, de ouders meer interesse hadden in dergelijke testen. De vraag rijst dan ook of de resultaten ouders met stress en/of zorgen voldoende gerust zouden stellen of dat het meer gaat om het concreet uiting geven aan de angstgevoelens. Er is veel debat over het zogenaamde 'recht op een open toekomst' van kinderen. Deterministische interpretaties van uitkomsten van erfelijkheidsonderzoek zouden dit mogelijk in de weg kunnen staan (Hens *et al.*, 2011). Nu wordt moleculair genetisch onderzoek vooral gedaan in de context van wetenschappelijk onderzoek om inzicht te krijgen in de pathofysiologie van psychiatrische stoornissen, maar deze methoden kunnen ook al gebruikt worden in een diagnostische setting in hele specifieke scenario's waarbij er sprake is van diagnostiseerbare genetische syndromen met complexe psychopathologie in combinatie met neurologische of andere somatische klachten en dysmorfieën. In de psychiatrische praktijk is dit slechts een kleine fractie van het totaal aantal patiënten.

Mensen die zelf een (mogelijk) erfelijke aandoening hebben of familieleden of kinderen hebben met een (mogelijke) erfelijke aandoening, kunnen worden verwezen naar een klinisch geneticus als zij vragen hebben over hun eigen risico, het risico voor (toekomstige) kinderen of voor verwanten. Een klinisch geneticus kan de familie in kaart brengen, medische gegevens opvragen van de aangedane verwanten om een gedetailleerd overzicht te maken van de familie en de aandoeningen die in de familie voorkomen. Aanvullend klinisch of laboratoriumonderzoek kan worden verricht om een mogelijke oorzaak op te sporen om zo een goede inschatting te geven van het risico op psychopathologie voor kinderen of andere verwanten. Indien er geen oorzaak wordt gevonden kan gebruik gemaakt worden van empirische gegevens om adviesvragers te informeren over het mogelijke (herhalings)risico (Austin and Peay, 2006). Genetische counseling kan adviesvragers helpen de best passende beslissing te nemen ten aanzien van etiologisch onderzoek, een kinderwens of het informeren van kinderen en andere verwanten. Eerder onderzoek wees uit dat patiënten met psychiatrische stoornissen positief staan tegenover een klinisch genetisch consult en de informatie die zij hier krijgen, ondanks de deels blijvende onzekerheid over de etiologie en het herhalingsrisico (Hippman *et al.*, 2013).

Conclusie

Psychiatrische stoornissen, en in het bijzonder de psychotische stoornissen, zijn sterk erfelijk bepaald en beïnvloeden ook de naasten van de patiënt. De erfelijke belasting heeft tot gevolg

dat er bij een individuele patiënt vaak meer familieleden zijn met soortgelijke psychiatrische klachten. Dit heeft grote effecten op het systeem die niet altijd duidelijk worden door de focus op een individuele patiënt in de behandeling. Ernstige psychiatrische klachten bij de ouder bemoeilijken de hechting van ouder en kind. Hier dient in de behandeling aandacht aan besteed te worden. Wanneer het kind eventueel psychopathologie ontwikkelt, kan de acceptatie hiervan gecompliceerd zijn. Aanvullende psychologische interventies kunnen hier, naast farmacotherapie, uitkomst bieden. Kinderen van patiënten met psychiatrische problematiek voelen zich onvoldoende gehoord gedurende de behandeling van hun ouder. Zij dienen op een adequate wijze te worden voorgelicht over wat er bij hun ouder aan de hand is. Ter preventie van het ontwikkelen van psychopathologie bij de kinderen is het beter de kinderen hierover voldoende te informeren en hen waar nodig te steunen. Ook zou hun ontwikkeling in de gaten gehouden moeten worden en is verwijzing naar de specialistische GGZ te overwegen. Zo kan de veerkracht worden bevorderd en wordt de kans op transgenerationele overdracht mogelijk beperkt. Er is steeds meer kennis beschikbaar ten aanzien van de moleculair-genetische oorzaken van psychiatrische klachten. Erfelijkheidsonderzoek bij patiënten en families waar veel psychiatrische klachten voorkomen is het overwegen waard en wordt gewaardeerd. Moleculair-genetische diagnostiek is echter op dit moment in veel gevallen nog niet informatief.

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9 |

General discussion

General discussion

Human genetic studies applying the classic Mendelian laws of inheritance have been performed since the beginning of the 20th century.[1] Much of the progress in human molecular genetics has been paced by technological developments.[2] In this thesis, we describe state-of-the-art family-based genetic analyses in order to identify the Mendelian risk factors underlying the phenotypes.[3] We show that it is possible, by combining the classical strategy of families-based linkage analysis with the next generation sequencing (NGS) technologies (exome sequencing and whole-genome sequencing), to detect genetic variants which segregate with the disease in families and provide converging evidence for their plausible role in disease aetiology.

In the first section focussing on adult-onset psychiatric disorders, we show that mutations in the gene *CSPG4* underlie rare forms of schizophrenia, which might provide insight in the general pathophysiology of the group of disorders that we currently label with the term schizophrenia. The protein encoded by *CSPG4*, called neuroglia2 (NG2), is involved in the development of the white matter in the brain. We present several lines of evidence that rare genetic coding variants in *CSPG4* are associated with the development of adult-onset schizophrenia. Similarly, we present in chapter two a family with bipolar disorder with a segregating missense variant in the gene *GRM2* which encodes the metabotropic glutamate receptor, type 2 protein (mGluR2). Through Sanger-based validation screening, we were able to identify additional rare missense variants in Dutch patients with bipolar disorder. We present functional evidence showing the identified variant has a loss-of-function effect. Overexpression of the variant resulted in a reduced functional effect compared to the normal protein. Furthermore, a large body of evidence in the literature demonstrates the mGluR2 is involved in the vulnerability for psychosis. Moreover, several large pharmaceutical companies have drug development programs specifically for the metabotropic glutamate signalling pathways.[4,5]

In the second section dealing with neurodevelopmental disorders, we present two families in which we found disease-causing candidate variants compatible with autosomal recessive inheritance. In a Dutch family with multiple children with autism spectrum disorders (ASD), we identified compound heterozygous variants in the gene *SLC39A7* coding for the intracellular zinc transporter ZIP7. Our own and existing functional work on zebrafish demonstrates that this gene is highly expressed in the central nervous system and especially

during early neural development.[6] Disruption of this gene in zebrafish causes a strong neurodevelopmental phenotype. Other variants in paralogue zinc transporter genes have previously been implicated in neurodevelopmental disorders, namely ASD.[7] Furthermore, in a northern Israeli family from Arab Bedouin origin we identified a homozygous mutation in the gene *ACO2* that encodes the mitochondrial aconitase protein (ACO2) and that segregates with hereditary spastic paraplegia, complicated with intellectual disability and microcephaly. For HSP, the genetic underpinnings and pathophysiology are much better understood than for most of the major psychiatric disorders. Yet, to our knowledge, *ACO2* has not previously been implicated in HSP, while it has been in other complicated movement disorders and ophthalmological disorders.[8] Additionally, the function of mitochondrial aconitase is in line with evidence from other forms of complicated HSP which are also caused by impaired mitochondrial functioning, such as SPG7 and SPG13.

In the last section on genomic rearrangements and syndromic patients, we present a family with bipolar disorder and a disease-segregating balanced translocation disrupting two genes: *BCL2L10* and *PNLDC1*. Furthermore, we show that with a clinical genetics consultation and standard genetic testing one can identify genomic aberrations in 24% of patients with syndromic forms of psychiatric disorders.

Family genetic studies complementary to large scale case/control studies

Having performed the work presented in this thesis, it became apparent that the study of multiplex families has its merits and is complementary to case/control cohorts such as GWAS studies or case/control exome studies. Our results indicate that Mendelian genetics is far from passé, a view which is supported by others.[9] By focusing on coding variants in a family-based design, it is possible to identify highly-penetrant rare segregating coding variants which underlie the psychopathology in those families. The possibility to perform segregation analysis in a pedigree provides a very nice opportunity to provide evidence for pathogenicity.[10] In large-scale case/control studies using next generation sequencing, a similar result has been observed as with the past microarray-based GWAS studies. So many variants are tested in an unbiased fashion that immense statistical power is required in order to reveal truly associated variants.[11–13] From a pragmatic standpoint, the question is whether or not that is a desirable approach. The success of the Mendelian Genetics Consortium shows that linkage analysis coupled with exome sequencing is valid approach for the identification of medically relevant

variants in families.[9] Such variants can instantaneously be used in a diagnostic genetic laboratory in diagnostic efforts for other patients and their families.

Understanding of disease aetiology in neurology is derived from family studies – Why should psychiatric disorders be any different?

If the purpose is to understand the psychopathology of psychiatric and neurodevelopmental disorders in general, we might have to look at other very comparable fields of study, such as neurology.[14] A profound problem in the study of the pathophysiology of psychiatric disorders is the absence of the opportunity to sample the organ of interest during pathological functioning. Currently no objective laboratory test exist that can reliably diagnose a patient with a psychiatric disorder. As well, the chance to find abnormalities on standard laboratory blood screening is very low.[15] This means that usually a diagnosis in psychiatry is based on clinical interviews and behavioural observations, as has been the case for many years. The nearest neighbour in this medical-scientific landscape of genetic research is the field of neurology, once a joint field with psychiatry. The genetic underpinnings of the neurodegenerative diseases are also far from being completely known.[16–18] In their core, these neurological disorders are comparable to psychiatric disorders although there are also distinctions: they both involve the brain, are most often only clinically diagnosed, and availability of brain imaging confirmation depends on treatment centre and country of treatment. Often, an improvement in symptoms in reaction to the pharmacological compound such as levodopa/carbidopa for Parkinson's disease is considered a confirmation of the diagnosis. Interestingly, the known genes which underlie the neuropathology in the major neurodegenerative disorders were identified through family-based studies.[19–22] After that, also unrelated patients were found to have causative less deleterious mutations in these genes and even environmental influences could alter the expression levels of these genes leading to an increased risk of developing clinical symptoms.[23] Recently, there has been renewed interest in the distinction between neurology and psychiatry. Neuroimaging studies have revealed different structures in the brain to be associated with pure neurological or pure psychiatric disorders whereby the neuroimaging abnormalities were found in the basal ganglia and temporal cortex whereas in the psychiatric cases, the abnormalities were more prominent in medial-prefrontal areas.[24] However, this distinction is put into perspective in an editorial in the same issue of the journal. There, it was said that, although there might be subtle differences at the level of neuroimaging, they both involve the same organ.[25] The distinction between

psychiatry and neurology based on the presence or absence respectively of a 'reliably associated and recognisable neuropathology process',[25] is likely not the best manner to distinguish the field of brain study.

More families need to be examined to sketch the landscape of mutations that may result in neuropsychiatric disorders

The question might then arise how many families would need to be investigated in order to identify the array of possibilities regarding genetic risk of neurodevelopmental and psychiatric disorders. The theoretical number of possible coding sequence alterations which might lead to pathological brain functioning is very large, and probably it will turn out the real number of possibilities will be very large too. The answer remains elusive until the experiments actually are performed. Statistical geneticist Kenneth Kendler indicates in his review in Molecular Psychiatry a few years ago, there must be some logical organisation. It might be complex, but the scenarios that there are either only one or two genetic forms of a certain diagnostic entity, or the scenario that every patients has a completely independent genetic vulnerability are unlikely.[26] It is more likely that there is an unknown, but finite, number of genetic variation that underlies clinical psychiatric phenotypes. In other disorders where the number of loci is still increasing, there appears to be convergence on a limited number of pathways, such as is currently the case for hereditary spastic paraplegia where there are >70 loci known which converge on <10 pathways.[27] For psychiatric disorders it might well be the same.[26]

The genetic architecture of childhood onset versus adult onset psychiatric disorders

It is likely that disease risk is inherently polygenic, meaning that the absolute disease risk is a function of the sum of both risk and protective alleles.[28] It might be that the more deleterious the type of mutations are, the earlier and more severe the onset is. This is the case with childhood-onset schizophrenia where the age of onset is before the age of 13,[29] and consistent with the more severe phenotypes in the syndromic cases described in chapter seven compared to the families described in the other chapters of this thesis. More generally, this is what has been found in other brain disorders such as Parkinson's disease, with a higher likelihood to have an identifiable genetic origin with earlier onset compared to typical late-onset disease.[16,30] It seems that the more severe the disorder, and the earlier the age of onset, the higher the likelihood that a highly penetrant mutation lies at the foundation of the phenotype. In detailed

studies of mouse models of cone-rod-homeobox protein CRX associated retinopaties this has been confirmed by determining the gene expression levels of *Crx* by RNA sequencing and correlating them with the phenotypic severity. There was a significant association between the reduction in the level of gene expression and the severity of the phenotype. Also, it was found there was a threshold above which, no apparent phenotype was observed.[31]

Genetic testing in the psychiatric clinic in a subgroup of complex patients

The work presented in this thesis is the result of genetic studies in the context of research. In chapter seven, we demonstrate that in a clinical setting with currently available diagnostic techniques, a causative or likely causative rare genomic rearrangement can be diagnosed in 24% of the syndromic psychiatric patients. Increasingly, next generation sequencing techniques are finding their way into clinical practice. Already now, we would suggest implementing genetic counselling and testing in syndromic cases. The current diagnostic yield in non-syndromic patients is too low to justify genetic testing in all non-syndromic psychiatric patients, although other opinions exist.[32] However, given the result from our study, we advise to start offering standardized genetic counselling and testing to all syndromic psychiatric patients and their families. The opportunity for genetic counselling and testing already exists for patients with neurodevelopmental phenotypes and their families. The diagnostic yield is high in these cases. Exome sequencing for example in intellectual developmental disorder yielded a diagnosis in 28 of 41 probands and in 18 (44%) a treatment was started specifically aimed at the identified dysfunction.[33]

Clinicians working in psychiatry should be trained in human molecular genetics

Genetic studies have been always performed in psychiatry, but always by experts and usually in the context of research. Initially, genetic research consisted primarily of statistical genetics in the form of epidemiology and twin studies. The figures such as concordance rates and population risk which were calculated in the early 20th century still hold to this day.[13,34] Dedicated researchers also applied rapidly the emerging molecular genetic techniques to the field of psychiatry. However, in the absence of clear-cut genetic findings, knowledge about the methodology of genetics never became common knowledge amongst clinicians in the field. Now increasingly, medicine is becoming the domain of the technologists with the continuous introduction of novel techniques to diagnose patients in a better way and at an earlier point in

their disease course. Human molecular genetic techniques are increasingly becoming mainstream techniques to diagnose human disease, indicate therapeutic opportunities, improve pharmacotherapy, and to determine the presence of hereditary risk factors. Clinicians working in psychiatry should have a better understanding of the available molecular genetic techniques and be able to assess the added value for patient care if the question arises.

Genetics might change the classification system of psychiatric disorders based on the molecular disturbance rather than the clinical phenotype per se

Historically, psychiatric disorders were classified by means of their phenomenology. The current methods of human genetics may reshuffle the current system of classification of psychiatric illness, since genes do not adhere to man-made nosology. Already in the recent cross-disorder genetic studies (where different diagnoses are pooled together), it has been observed that certain genetic risk factors influence the risk of multiple psychiatric disorders.[35] With the collective years of experience in the field of psychiatric genetics, we have learned that genes are often not solely responsible for a given function, but instead affected an entire biological cascade of events which leads to a vulnerability at a systems level.[26]

Concluding remarks

The families and patients described in this thesis agreed to actively participate in our genetic and cellular studies of the disorders from which they suffer. Their contribution was instrumental in allowing us to make an effort to better understand these disorders and advance the knowledge in the field. Our results show that there are identifiable genetic factors that are associated with an increased probability of developing psychiatric illness. In concert with other risk factors in the environment, they can result in manifestation of the phenotype. Our work so far is still the beginning of the landscape of possibilities with regard to the number of genetic variants that convey a relatively strong genetic risk for illness manifestation. A lot of work remains ahead to identify other major genetic risk factors that predispose to psychiatric illness, to model how the genetic architecture underlies clinical phenotypes, and how we can use this newly acquired information in the discovery of therapeutic agents and apply these to patient care and cure.

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Appendices

Summary

Psychiatric disorders comprise a diverse class of severely disabling conditions that profoundly affect the quality of life of the patients and their family members. The work presented here concerns the genetic and functional-biological analysis of families with a high incidence of adult psychiatric or neurodevelopmental disorders.

In **chapter one** of this thesis, we describe the state of the art in psychiatry with regard to the phenotypic classification, neurobiological and genetic underpinnings, as well as the place of genetics and the concept of heritability in the field of psychiatry. We also provide an overview of the different techniques in the field of human genetics. As in most fields of medicine, scientific progress is intimately linked to technological development.

The two subsequent chapters describe our work in two hallmark psychiatric disorders schizophrenia and bipolar disorder. These disorders are considered life-long and require chronic pharmacotherapy and often hospitalizations. In **chapter two**, we describe a Dutch family with a high incidence of schizophrenia. Through linkage analysis coupled with exome sequencing, we identified a segregating missense mutation in the gene *CSPG4*. In screening other families with schizophrenia, we identified multiple other rare coding variants segregating with disease. We identified one variant segregating in two independent Mexican families and this variant was also associated with schizophrenia in Sweden. In combination with brain imaging and induced pluripotent stem cell-derived cell culture experiments (showing impaired functioning of the oligodendrocyte precursor cells), our results implicate *CSPG4* in the vulnerability for developing schizophrenia.

In **chapter three**, we describe the identification of a rare missense variant in the gene *GRM2* segregating in a Dutch family with bipolar disorder. We present preliminary functional evidence showing that the variant has a loss-of-function effect most likely caused by reduced expression of the receptor at the cell membrane and reduced heterodimerization between the mGluR2 receptor and the serotonin 2A receptor (5HT2A).

Next, we describe two families with neurodevelopmental phenotypes. Also, here we were able to identify rare genetic variants that affect the coding sequence of genes thought to underlie their respective phenotypes. In **chapter four**, we describe a Dutch family in which four of the

five siblings have autism spectrum disorder. We identified compound heterozygous variants in the gene *SLC39A7*, encoding ZIP7, a protein that transports zinc from the endoplasmatic reticulum to the cytoplasm. Variants in the gene *SLC30A5* that codes for the zinc transporter ZnT5 had been implicated before in ASD. ZIP7 is highly expressed in brain during early development. Preliminary functional data indicate a possible loss-of-function mechanism for both of the identified variants. Others showed that knockdown of ZIP7 causes significant neurodevelopmental phenotypes in the zebra fish, which could be restored by adding zinc to the swimming water.

In **chapter five**, we describe a consanguineous Israeli family from Arab-Bedouin descent where in the youngest generation, two children suffering from childhood-onset complicated hereditary spastic paraplegia with intellectual disability, and microcephaly. Through homozygosity mapping and exome sequencing, we were able to identify a rare missense mutation in the gene *ACO2* that codes for mitochondrial aconitase. This is an essential enzyme in the tricarboxylic acid cycle (TCA cycle) transforming citrate to isocitrate. Mutations in *ACO2* have not yet been described to result in complicated hereditary spastic paraplegia, but have been found to cause other syndromal complicated movement disorders. Dysfunction of the mitochondrial respiration chain has been found to be implicated in complicated hereditary spastic paraplegia caused by other genetic defects.

In the third section, we describe a patient cohort and a family with larger genomic abberations, which are associated with complicated psychiatric syndromes.
In **chapter six**, we describe a Dutch family with a balanced translocation segregating with bipolar disorder. Through karyotyping, fluorescent in-situ hybridization and whole genome sequencing, we were able to identify the exact breakpoints of the translocation. We found both breakpoints were intragenic and disrupted the genes *BCL2L10* and *PNLDC1*. We deem BCL2L10 likely to be associated with the disease risk since it codes for an anti-apoptotic protein in the mitochondrial death pathway. We propose these genes as candidate genes for bipolar disorder.

In **chapter seven**, we describe the outcomes of a diagnostic project in peripheral inpatient psychiatric hospitals where patients were in we identified copy number variants (CNVs) in substantial fraction of the patients screened. In our view, this justifies microarray-based

structural variant screening to be offered to patients and their family members in certain clinical scenarios in which there is not just the presence of a psychiatric disorder, but also intellectual disability and/or multiple congenital abnormalities.

Chapter eight is an essay regarding the psychodynamics in families affected by severe psychiatric illness. Here we discuss the psychological effects of growing up in a family with a high incidence of mental illness.

In **chapter nine**, we discuss our findings in the context of the current state of the art of psychiatric genetics and the future developments in this field.

Samenvatting

Psychiatrische stoornissen omvatten een brede groep sterk invaliderende aandoeningen die de kwaliteit van leven van de patiënt en zijn of haar naasten sterk negatief beïnvloedt. Dit werk betreft de studie van de genetica en het functioneel-biologisch mechanisme in families waarin veel psychiatrische stoornissen of ontwikkelingsstoornissen voorkomen.

In **hoofdstuk één** van dit proefschrift beschrijven we de huidige stand van zaken en de visie in de psychiatrie ten aanzien van de classificatie van stoornissen, alsook de neuronale en genetische basis van deze stoornissen. Daarnaast beschrijven we de verschillende methoden die bruikbaar zijn bij de studie van de humane genetica. Zoals in de meeste specialismen van de geneeskunde is wetenschappelijke vooruitgang sterk verbonden met technologische ontwikkeling.

In de twee daaropvolgende hoofdstukken bespreken we ons werk ten aanzien van de twee prototype psychiatrische stoornissen schizofrenie en bipolaire stoornis. Beide stoornissen worden gezien als chronisch en vereisen levenslange farmacotherapie en vaak ook opnamen in een psychiatrisch ziekenhuis. In **hoofdstuk twee** beschrijven we een Nederlandse familie waarin veel schizofrenie voorkomt. Met *linkage analyse* en *whole-exome sequencing* waren we in staat een segregerende mutatie in het gen *CSPG4* te identificeren. Bij het screenen van andere families met schizofrenie voor mutaties in dit gen vonden we meerdere andere zeldzame segregerende varianten die ook een verandering in het eiwit teweegbrengen. We vonden onder andere een variant die segregeerde in twee onafhankelijke families en deze variant associeerde ook significant met schizofrenie in een Zweeds cohort met patiënten met schizofrenie en controles. In combinatie met beeldvormende technieken voor het brein en geïnduceerde pluripotente stamcelexperimenten lieten we dysfunctie zien van de oligodendrocyt precursorcellen. Dit tezamen impliceert dysfunctie van *CSPG4* en het eiwit waarvoor het codeert (NG2) in de kwetsbaarheid voor het ontwikkelen van schizofrenie.

In **hoofdstuk drie** beschrijven we de identificatie van een zeldzame genetische variant in het gen *GRM2* in een Nederlandse familie met bipolaire stoornis. We laten functioneel bewijs zien dat de geïdentificeerde variant resulteert in functieverlies van het eiwit dat hoogstwaarschijnlijk wordt veroorzaakt door verminderde expressie van de metabotrope glutamaat receptor, type 2

(mGluR2) op het celmembraan. Dit leidt mogelijk tot verminderde heterodimerisatie van de mGluR2 receptor en de serotonine 2A receptor (5-HT2AR).

Hierna beschrijven we twee families met ontwikkelingsstoornissen. Ook hier konden we zeldzame genetische varianten identificeren die de eiwit-coderende sequentie veranderen van genen waarvan gedacht wordt dat ze ten grondslag liggen aan het respectievelijk fenotype. In **hoofdstuk vier** beschrijven we een consanguïne Nederlandse familie waarin vier van de vijf kinderen belast waren met een stoornis in het autisme spectrum (ASS). We vonden een samengestelde (*compound*) heterozygote mutatie in het gen *SLC39A7* dat codeert voor het eiwit ZIP7. Dit eiwit transporteert zink vanuit het endoplasmatisch reticulum (ER) naar het cytoplasma. Varianten in het gen *SLC30A5,* dat codeert voor de zinktransporter ZnT5, dat op zijn beurt zink transporteert van cytoplasma naar het ER, zijn eerder geassocieerd met ASS. Het eiwit ZIP7 komt sterk tot expressie in de hersenen gedurende de vroege ontwikkeling. Preliminare functionele experimenten laten effecten van beide varianten zien die compatibel zijn met een verlies van functie van het eiwit. Andere onderzoekers lieten zien dat reductie van de expressie van ZIP7 in zebravissen resulteerde in een sterk verslechterde neuronale ontwikkeling. Dit kon worden hersteld door zink aan het zwemwater toe te voegen.

In **hoofdstuk vijf** beschrijven we een consanguïne Arabisch-Bedouine familie uit Israël. Twee van de vier kinderen hadden gecompliceerde erfelijke spastische paraplegie met microcefalie en zwakbegaafdheid. Door middel van het in kaart brengen van de homozygote regio's (*homozygosity mapping*) en het aflezen van alle eiwit-coderende delen van het DNA (*exome sequencing)* konden we een zeldzame genetische variant vaststellen in het gen *ACO2* dat codeert voor mitochondriële aconitase. Dit is een essentieel enzym in de citroenzuurcyclus en transformeert citraat naar isocitraat. Mutaties in *ACO2* zijn niet eerder beschreven bij gecompliceerde erfelijke spastische paraplegie, maar wel bij andere complexe bewegingsstoornissen. Daarnaast is dysfunctie van de mitochondriële ademhalingsketen eerder geïmpliceerd in gecompliceerde erfelijke spastische paraplegie.

In het derde deel van dit proefschrift beschrijven we een cohort patiënten en een familie met grotere genomische mutaties, die geassocieerd leken te zijn met complexe psychiatrische problematiek. In **hoofdstuk zes** beschrijven we een Nederlandse familie met een gebalanceerde chromosomale translocatie die segregeert met bipolaire stoornis. Door middel

van karyotypering en fluoriserende in-situ hybridisatie en *whole genome sequening* konden we de precieze breekpunten van de translocatie vaststellen. Beide breekpunten waren intrageen en verstoorden de genen *BCL2L10* en *PNLDC1*. Op basis van de literatuur stellen wij *BCL2L10* voor als kandidaat-gen voor bipolaire stoornis. Dit gen codeert voor een anti-apoptotisch eiwit in de mitochondriële apoptose cascade.

In **hoofdstuk zeven** beschrijven we de uitkomsten van een diagnostiekproject van syndromale patiënten in twee perifere psychiatrische centra. We vonden causale of mogelijk causale dosisvarianten (*copy number variants*; CNVs) in een substantieel deel van de patiënten. Ons inziens rechtvaardigt deze bevinding genetisch-diagnostische screening van patiënten met een dergelijke syndromale presentatie en hun familieleden.

Hoofdstuk acht is een essay over de psychodynamiek in families die sterk belast zijn met psychiatrische stoornissen. Hierin bespreken we de psychologische effecten van het opgroeien in een gezin waarin psychiatrische stoornissen voorkomen.

In **hoofdstuk negen** bespreken we onze bevindingen in de context van de huidige literatuur en de ontwikkelingen in het veld.

Curriculum Vitae

Christian Bouwkamp was born on July 8, 1987 in Delfzijl. He graduated from secondary school in 2004 at the Augustinus College in Groningen, after which he obtained the propaedeutic diploma in Aircraft Engineering from the Hogeschool van Amsterdam. From 2005 to 2010, Christian studied Clinical Psychology at Leiden University. He performed his clinical training at the Clinical Centre of Acute Psychiatry at the Parnassia Psychiatric Centre in The Hague and completed his thesis research on the evaluation of Interpersonal and Social Rhythm Therapy for patients with bipolar disorder at the Department of Mood Disorders at PsyQ in The Hague. In 2009, he commenced a research master at the Department of Neurosciences at the Erasmus University Medical Centre in Rotterdam. In September 2011, he started his PhD research project at the Department of Psychiatry and Clinical Genetics at the Erasmus University Medical Centre in Rotterdam.

PhD portfolio

Name PhD student: Christian Bouwkamp
Erasmus MC department of Psychiatry & department of Clinical Genetics

PhD training

Courses	**Year**	**Workload (h)**
SNPs and human disease	2010	40
Positive and Negative Symptoms Scale, Parnassia Academy, The Hague	2010	7
EBI-Wellcome Trust Bioinformatics Summer School, Hinxton, UK	2012	40
Teach the Teacher, didactic skills course	2015	16
English biomedical writing & communication, Erasmus MC	2015	112
Medical-scientific ethics and integrity course, Erasmus MC	2015	8

Presentations and conferences		
Voorjaarscongres Nederlandse Vereniging voor Psychiatrie, workshop	2010	8
Voorjaarscongres Nederlandse Vereniging voor Psychiatrie, poster presentation	2013	1
International Society of Psychiatric Genetics Meeting, Boston, poster presentation	2013	40
American Society of Human Genetics Meeting, Boston, poster presentation	2013	40
American Society of Human Genetics Meeting, Baltimore, poster presentation	2015	40

Teaching Activities

Lecturing	**Year**	**Work load (h)**
Neuroscience master module MNEU-1.9 Psychiatric Disorders	2013-2016	15
Neuroscience Minor - Psychiatric Disorders	2014	5
Kennismaking met de beroepspraktijk – medical curriculum	2014	15
Elective course in psychiatry - medical curriculum	2012-2016	50
Minor Psychiatry - medical curriculum	2010	5
Thema Ba.3.A Overerving in de praktijk	2014-2016	10

Supervision	**Year**	**Period**
Premika Boedhoe, BSc. Student	2013	3 months
Ineke Maaskant, MSc. Student	2015	9 months

Publication list

Bouwkamp CG, Kievit AJ, Olgiati S, Breedveld GJ, Coesmans M, Bonifati V, Kushner SA. (2016). A balanced translocation disrupting BCL2L10 and PNLDC1 segregates with affective psychosis. Am J Med Genet B Neuropsychiatr Genet. doi: 10.1002/ajmg.b.32465. [Epub ahead of print]

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