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ORIGINAL ARTICLE

CNTNAP2 gene dosage variation is associated with schizophrenia and epilepsy

JI Friedman[1,8], T Vrijenhoek[2,8], S Markx[3], IM Janssen[2], WA van der Vliet[2], BHW Faas[2], NV Knoers[2], W Cahn[4,5], RS Kahn[4,5], L Edelmann[1], KL Davis[1], JM Silverman[1], HG Brunner[2], A Geurts van Kessel[2], C Wijmenga[6,7], RA Ophoff[5,6] and JA Veltman[2]

[1]*Departments of Psychiatry and Human Genetics, Mount Sinai School of Medicine, New York, NY, USA;* [2]*Department of Human Genetics, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands;* [3]*Department of Psychiatry, Columbia University, New York, NY, USA;* [4]*Department of Psychiatry, Utrecht University, Utrecht, The Netherlands;* [5]*Rudolf Magnus Institute of Neuroscience, Utrecht University, Utrecht, The Netherlands;* [6]*Department of Medical Genetics, Utrecht University, Utrecht, The Netherlands and* [7]*Department of Genetics, University Medical Centre Groningen, Groningen, The Netherlands*

A homozygous mutation of the *CNTNAP2* gene has been associated with a syndrome of focal epilepsy, mental retardation, language regression and other neuropsychiatric problems in children of the Old Order Amish community. Here we report genomic rearrangements resulting in haploinsufficiency of the *CNTNAP2* gene in association with epilepsy and schizophrenia. Genomic deletions of varying sizes affecting the *CNTNAP2* gene were identified in three non-related Caucasian patients. In contrast, we did not observe any dosage variation for this gene in 512 healthy controls. Moreover, this genomic region has not been identified as showing large-scale copy number variation. Our data thus confirm an association of *CNTNAP2* to epilepsy outside the Old Order Amish population and suggest that dosage alteration of this gene may lead to a complex phenotype of schizophrenia, epilepsy and cognitive impairment.
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Introduction

Investigations of complex brain disorders such as schizophrenia (MIM 181500) and epilepsy have demonstrated considerable heterogeneity of the genetic and pathophysiological components of these illnesses. Moreover, the phenotypic manifestations of schizophrenia are numerous and variable, and may include delusions, disordered thought, hallucinations, negative symptoms, and cognitive and functional impairment.[1] In addition, many distinct epilepsy syndromes do exist, which are defined by seizure characteristics, location and age of onset. These factors have complicated the identification of genes and their products that serve as vulnerability factors for these disorders. Linkage and association studies have yielded several candidate genes for schizophrenia[2,3] and epilepsy,[4] but despite these successes the genetic mechanisms underlying the comprehensive pathophysiology of these brain disorders remain poorly understood.

Technical developments in microarray-based genome profiling have facilitated the routine genome-wide detection of genomic copy number variation (CNV) with a resolution of up to ~100 kb.[5,6] As a result, subtle CNVs have been linked to the pathogenesis of a number of diseases, ranging from rare monogenic to complex disorders.[7–14] Moreover, limited evidence suggests a role for CNVs in the susceptibility to psychiatric disease such as schizophrenia.[15]

In the current study, we used both genome-wide and targeted genomic approaches to investigate CNVs in patients with epilepsy and schizophrenia. These approaches revealed the presence of unique CNVs on chromosome 7q34–36.1 in three unrelated patients with epilepsy and schizophrenia, all affecting the *CNTNAP2* gene. *CNTNAP2* variation has previously been associated with epilepsy,[16] but not with schizophrenia. *CNTNAP2* encodes Caspr2 (contactin-associated protein 2), a member of the neurexin superfamily, a group of transmembrane proteins that mediate cell–cell interactions in the nervous system. Caspr2 is predominantly expressed in the nervous system in the juxtaparanodal region of axons of myelinated neurons and in oligodendrocytes.[17]

Correspondence: Dr JA Veltman, Department of Human Genetics, Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen Medical Center, Geert Grooteplein 10, PO Box 9101, 6500 HB Nijmegen, The Netherlands.
E-mail: j.veltman@antrg.umcn.nl
[8]These authors contributed equally to this work.
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Caspr2 is essential for the localization of voltage-activated K^+ channels in the juxtaparanodal region of axons, which may function to stabilize conduction and help to maintain the intermodal resting potential.[18] Targeted disruption of Caspr2 results in marked reduction in the accumulation of K^+ channels at the juxtaparanodes in both central and peripheral nervous systems.[19]

This is the first study demonstrating an association between the *CNTNAP2* gene and schizophrenia. Moreover, this study is the first to demonstrate the presence of dosage variations at this gene, resulting in both epilepsy and schizophrenia.

Materials and methods

Patient material

Genomic DNA from ethylenediaminetetraacetic acid venous blood was obtained from three independent sources. Patient 1 was brought to the diagnostics department at the Radboud University Nÿmegen Medical Center for routine karyotyping with a clinical presentation of severe mental retardation, epilepsy and language development problems among other findings (see Table 1 for details). Patient 2, who presented with schizophrenia and epilepsy, was identified from a cohort of 23 patients with syndromal schizophrenia (for example schizophrenia accompanied by numerous minor physical anomalies, mental retardation, seizure disorder and other medical problems) recruited from Pilgrim Psychiatric Center, W Brentwood, NY, USA, and referred to the Department of Human Genetics, Radboud University Nijmegen Medical Centre for array-based comparative genomic hybridization (array CGH) analysis. Patient 3 was identified from a cohort of 312 schizophrenic patients recruited at the Department of Psychiatry, Utrecht University, the Netherlands for targeted single nucleotide polymorphism (SNP) array analysis. These were analyzed together with 312 healthy individuals of Dutch origin as a control set. The cohorts were collected following institutional review board approval and after obtaining signed informed consent.

Array-based comparative genomic hybridization

Array CGH was performed as described previously[10] using a tiling-resolution BAC array. All data were mapped on the National Center for Biotechnology Information (NCBI) build 35 of the UCSC Genome Browser hg 17 (http://genome.ucsc.edu/).

Singe nucleotide polymorphism assay

To assess genetic variation in the genomic region around *CNTNAP2*, we developed a targeted SNP assay for the Illumina GoldenGate Bead Array (Illumina Inc., San Diego, CA, USA). The assay consisted of 194 haplotype-tagging SNPs in the *CNTNAP2* locus (chromosome 7: 145 444 787–148 582 400; NCBI build 35 of the UCSC Genome Browser hg17 (http://genome.ucsc.edu/); average spacing between two SNPs was 16 kb). We performed a straightforward χ^2 test for association of individual SNPs with schizophrenia. We also checked for copy number changes by simultaneous measurement of both signal intensity variations ($\log_2 R$) and changes in allelic composition.[20]

Multiplex ligation-dependent probe amplification

To confirm the microarray analysis, samples were screened with multiplex ligation-dependent probe amplification (MLPA), essentially as described previously.[21] MLPA probes were designed for exons 2, 3, 4, 5, 14, 24 (one probe for each exon) and intron 3 of *CNTNAP2* (three different probes), and for exon 3 of *PDIA4* and exon 14 of *NOS3* (one probe for each exon of both). Sequences of test and control probes are in Supplementary Table S1. Probes were developed in accordance with a protocol provided by MRC-Holland (Amsterdam, The Netherlands). Ten probes were combined in one MLPA assay, in combination with three standard control probes. Equal amounts of probe mix and hybridization buffer were added to 100–200 ng of DNA from each sample, followed by heat denaturation and overnight incubation at 60°C. Ligation products were amplified by regular PCR with the use of a 6-FAM-labeled primer set. Amplification products were identified and quantified by capillary electrophoresis on an ABI 3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Data were normalized by dividing each probe's signal strength by the average signal strength of the sample. This normalized peak pattern was divided by the average peak pattern of all samples in the same experiment. Values for control wild-type peaks were centered at 1.0. A copy number loss was defined as that below a fixed threshold value of 0.7.

Fluorescent in situ *hybridization analysis*

Fluorescent *in situ* hybridization (FISH) experiments were performed for *de novo* checking on metaphase spreads prepared from patient-derived lymphoblast cell lines using routine procedures. Probe labeling, slide preparation and hybridization were carried out as described elsewhere,[14] using probes listed in Supplementary Table S2.

Sequence analysis

To sequence all 24 exons of the *CNTNAP2* gene, we designed primers in the intronic region surrounding each exon using Primer3,[22] and performed standard PCR reactions. Primer sequences and conditions are in Supplementary Table S3.

Results

We identified a hemizygous deletion involving chromosome region 7q34–7q36.1 in patient 1 by routine chromosome analysis (data not shown). Patient 1 is a severely mentally retarded 21-year-old Caucasian male born to healthy non-consanguineous parents. No other family members were reported to have mental retardation or epilepsy. At the age of 2, the

Table 1 Overview of clinical characteristics of the three patients with a deletion in chromosome 7q34–7q36.1

	Patient 1	*Patient 2*	*Patient 3*
Deletion			
Size	11 Mb	1.5 Mb	220 kb
Number of genes	58	4	1
Inheritance	*De novo*	Unknown	Unknown; healthy sister does not show deletion
Patient details			
Gender	Male	Female	Female
Year of birth	1979	1955	1973
Ethnicity	Caucasian	Caucasian	Caucasian
Status at birth	Premature birth, severe respiratory problems, neonatal sepsis	Normal	Premature birth, dizygotic twin, severe respiratory problems
Family history			
Schizophrenia	—	—	Aunt with bipolar disorder
Epilepsy	—	—	Father's cousin with epilepsy
Physical illness	—	Mother and two maternal aunts with chronic severe mental illness	
Epilepsy			
Age of first seizure	2	34	16
Type	Focal right frontotemporal epilepsia with secondary generalization	General tonic-clonic seizure	Brainstem regulation disorder
EEG	Slow theta/delta	Dysrythmic theta/delta	Not determined
Therapy	Carbamazepine	Dilantin	None
Schizophrenia			
Age of onset	Could not be determined	18	21
Type		Paranoid	Paranoid
Other features			
Speech	Almost absent	Deteriorated	Normal
Cognitive abilities	Severe mental retardation	Progressive cognitive deterioration, IQ = 63 at ascertainment	Normal IQ
Additional features	Severely delayed psychomotor development Autistic features	Difficulties in communication Mild cerebral atrophy	

Abbreviations: EEG, electroencephalogram; IQ, intelligence quotient.

patient suffered from seizures, first noticed by the parents and later confirmed by electroencephalogram (EEG). Anticonvulsant medication (carbamazepine) was initiated and has remained necessary until today (Table 1). We confirmed this deletion by using both FISH and MLPA (Figure 1b). FISH analysis of both parents failed to reveal any chromosome 7 aberrations, indicating that the deletion occurred *de novo* in the patient. We also used tiling-resolution genome-wide array CGH to characterize the deletion in detail (Figure 1a). By doing so, we found this deletion to be 10.7 Mb in size and to encompass 58 known genes, of which only *EPHB6*, *EZH2* and *CNTNAP2* are expressed in the brain.

We identified a second hemizygous deletion involving *CNTNAP2* in patient 2 by tiling-resolution array CGH. This 1.5 Mb deletion was identified at the 7q35–7q36.1 locus and was found to encompass four genes: *CUL1*, *EZH2*, *PDIA4* and *CNTNAP2* (Figure 1c). The microdeletion was confirmed by MLPA analysis (Figure 1b). The patient is a 50-year-old Caucasian female diagnosed with schizophrenia (age of onset = 18), epilepsy and severe cognitive impairment as indicated by a WAIS III full-scale intelligence quotient (IQ) = 63 (Table 1). She completed 10 years of mainstream education and was assessed to have a premorbid IQ = 94 (derived from WRAT-R Reading score), indicating a significant decrement in cognitive ability subsequent to the onset of schizophrenia. She also experienced a significant deterioration in communicative and self-care abilities requiring prolonged institutionalization. Her mother and both of the

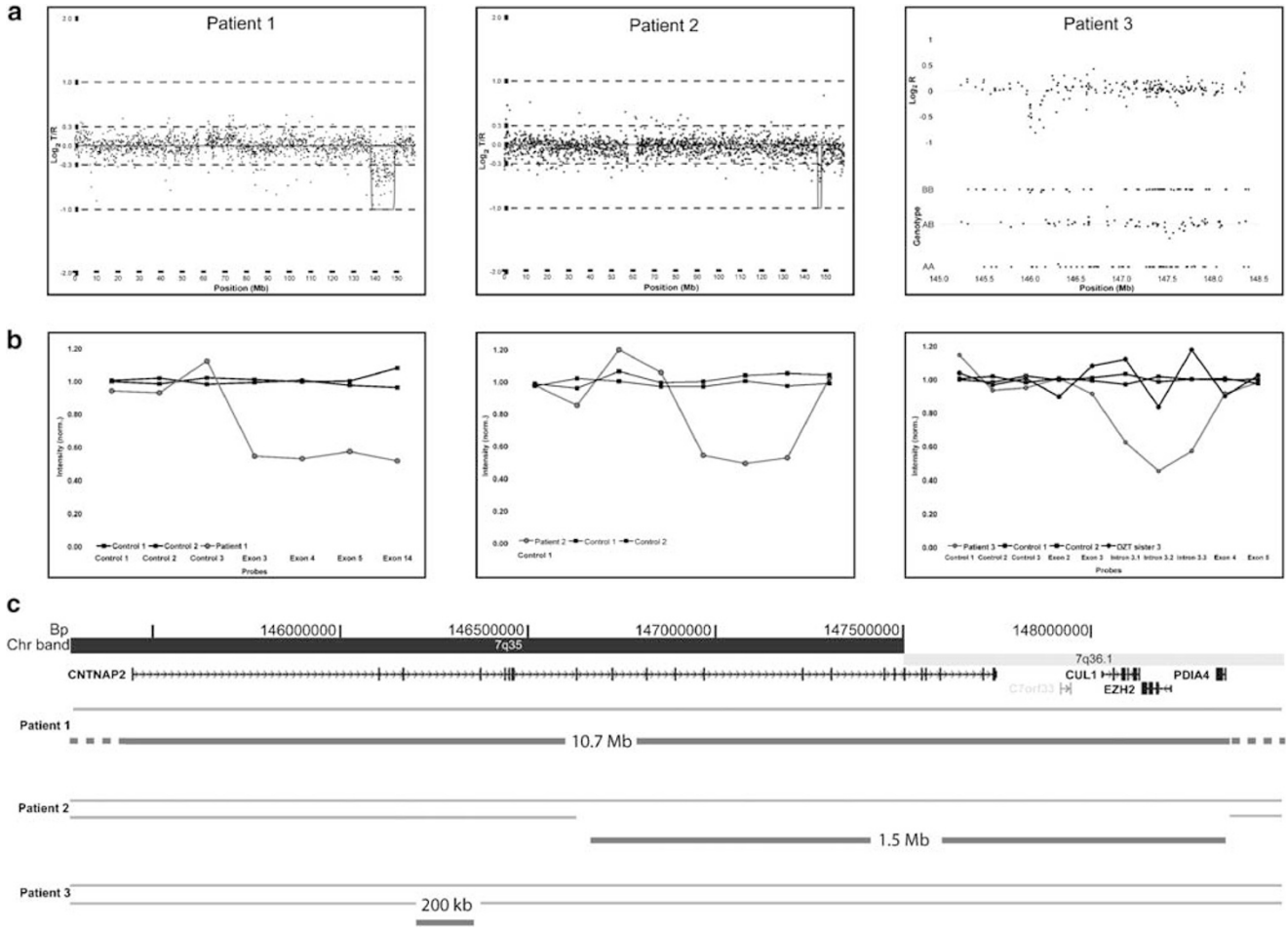

Figure 1 Genomic deletions of the *CNTNAP2* locus in chromosome 7 in three patients. (**a**) Microarray-based chromosome 7 profiles for patients 1–3. The profiles for patients 1 and 2 were obtained from tiling-path resolution genome-wide arrays containing 32 447 human BAC clones, indicated by small circles. These represent the log 2-transformed and normalized test-over-reference intensity ratios. The clones are ordered from pter – qter on the basis of physical-mapping positions obtained from NCBI build 35 of the UCSC genome browser hg17 http://genome.ucsc.edu/. The normalized ratios were analyzed for loss and gain regions by a standard hidden Markov model,[23] as indicated by the red line. The profiles show the 10.7 Mb deletion of chromosomal region 7q34–7q36.1 in patient 1, and the 1.5 Mb deletion of chromosomal region 7q35–7q36.1 in patient 2. The profile for patient 3 was obtained from an array that contained 194 haplotype-tagging SNPs in the *CNTNAP2* locus, indicated by small circles. In the upper profile, the circles represent the log 2-transformed total intensity ratio $\log_2 R$ of both variants of an SNP. In the lower profile, they represent the B-allele frequency of the SNPs. The 220 kb deletion in *CNTNAP2* is represented by a simultaneous decrease in $\log_2 R$ and shift from heterozygous to homozygous state of the SNPs. (**b**) MLPA confirmation of the deletions in *CNTNAP2* in all three patients. Different probe sets were used to confirm the deletions, which varied in size. (**c**) Schematic overview of the deletions in all three patients. For each patient, the deleted genomic region is shown in red. MLPA, multiplex ligation-dependent probe amplification; SNP, single nucleotide polymorphism.

mother's siblings suffered from unspecified chronic psychiatric illnesses requiring prolonged institutionalization. All three of this patient's male siblings were free from psychiatric illness. At age 34, the patient experienced her first and only-documented seizure, characterized as generalized tonic-clonic. Her EEG (16 channel) demonstrated slow dysrythmia in theta and occasional delta, predominantly in the frontal area (Table 1).

The above results prompted us to assess further genetic variation in the minimal deleted region using a targeted SNP assay. We genotyped the cohort of 312 patients with schizophrenia and a cohort of 312 Dutch control subjects for 194 haplotype-tagging SNPs in the *CNTNAP2* locus. The χ^2 test for association revealed no SNP to be significantly ($P < 0.05$) associated with schizophrenia. However, a detailed copy number analysis of all data revealed a hemizygous deletion involving *CNTNAP2* in one patient from the cohort of 312 schizophrenic patients (patient 3), represented by 15 consecutive homozygous SNPs with an average $\log_2 R$-value of −0.42 (Figure 1a). Subsequent MLPA analysis confirmed the deletion, which encompasses 220 kb and affects only intron 3 of the *CNTNAP2* gene (Figure 1b). The patient is a 32-year-old Caucasian female diagnosed

with schizophrenia who suffered from epileptic seizures starting from age 16. Her IQ is reported as normal. Family history revealed one aunt with bipolar disorder and a paternal cousin with epilepsy (Table 1). Analysis of the healthy dizygotic twin sister of the patient did not reveal any deletion at this locus (Figure 1b).

No CNVs larger than 10 kb have been reported in the 7q34–7q36.1 chromosomal region, in which all deletions observed in the present study occurred (see the Database of Genomic Variants; http://projects.tcag.ca/variation/). In addition, we did not observe any deletions in our cohort of 312 healthy controls tested on the targeted SNP array, nor in an additional cohort of 100 healthy individuals analyzed by MLPA. Moreover, no CNV has been observed at this genomic locus in more than 500 mentally retarded patients and 100 unaffected parental samples tested on tiling-resolution BAC arrays in the diagnostics laboratory at the Radboud University Nijmegen Medical Centre. Sequence analysis of all 24 exons of the *CNTNAP2* gene revealed no additional mutations in these three patients.

Discussion

Recently, a syndrome characterized by focal epilepsy, macrocephaly, diminished deep tendon reflexes, mental retardation and other neuropsychiatric disturbances (designated as cortical dysplasia-focal epilepsy syndrome) was described in association with a homozygous mutation of the *CNTNAP2* gene in Old Order Amish children.[16] The results of the present study demonstrate an association between dosage variation of the *CNTNAP2* gene and epilepsy outside the Old Order Amish community. Sequence analysis of all 24 exons of the *CNTNAP2* gene in all three patients revealed no mutation in the remaining allele, indicating that haploinsufficiency of this gene leads to the disease. Given that these deletions are not common events nor located in identified regions of high CNV,[5,6] these findings are unlikely to be coincidental. Moreover, unlike the neuropsychiatric presentation suggestive of a pervasive development disorder in the Amish children with a homozygous mutation of *CNTNAP2*,[16] two out of three subjects in the present study presented with a diagnosis of schizophrenia. To our knowledge, this is the first report of an association between the *CNTNAP2* gene and schizophrenia. However, it is noteworthy that a chromosomal translocation disrupting the *CNTNAP2* locus seems to result in Gilles de la Tourette syndrome (MIM 137580) and obsessive compulsive disorder,[24] although the causality of this translocation was recently questioned.[25] *CNTNAP2* plays a role in the organization of myelinated axons,[17,19] and its disruption affects neuroblast migration.[16] Interestingly, brains of schizophrenic patients show evidence for myelin-related dysfunction[26] and aberrant neuronal migration.[27,28] This may explain the observed association between *CNTNAP2* disruptions and susceptibility to schizophrenia.

Although the three patients in the present study showed moderate clinical overlap, they exhibited differences that may be related to the varying size of the deletion. The deletion in patient 1 affected 57 other genes in addition to *CNTNAP2*. The more severe phenotype observed in this patient as compared to the other two (for example severe mental retardation, multiple epileptic seizures, severely delayed psychomotor development) may be the result of an additive effect of one or more of these other genes. Because of the severe mental retardation and inability to communicate, a diagnosis of schizophrenia could not be determined in this patient. Similarly, the deletion in patient 2 encompasses four additional genes, among which are the Cullin-1 (*CUL1*) and Enhancer of Zeste Homolog 2 (*EZH2*) genes, which are involved in protein ubiquitination and histone methylation, respectively.[29,30] Although these genes have been reported to be only indirectly involved in brain function,[29,30] they might have also contributed to the phenotype of this patient. Patient 3 had the smallest deletion encompassing only intron 3 of the *CNTNAP2* gene, and presented with the least severe phenotype (normal IQ, normal speech and higher levels of functioning), which still included epilepsy and schizophrenia. Although no exons were affected in the *CNTNAP2* deletion found in patient 3, regulatory motifs within this region may have affected the expression of the *CNTNAP2* gene. Alternatively, there are examples of enhancers that act over long distances, affecting other genes than those in which they reside.[31] Further functional analysis of this intronic *CNTNAP2* deletion was not possible in this patient, owing to the restricted pattern of *CNTNAP2* expression in the nervous system.[17]

Our study and that of others[16] indicate that rare point mutations and/or dosage variation at the *CNTNAP2* gene can cause both neurological and psychiatric disorders. Examples of other rare mutations of genes resulting in variable expression of neurologic and psychiatric disease include mutations in the *KCNN3* gene[32] as well as the *SYNGR1* gene[33] in schizophrenia, and the association of rare variants of the *KCC3* gene with bipolar disorder.[34] On the other hand, CNV of genes in the 22q11 region, which results in velo-cardio-facial syndrome, produces developmental neurologic abnormalities and is one of the most common known genetic risk factors for the development of schizophrenia.[35]

In conclusion, this study confirms the recently reported association between the *CNTNAP2* gene and epilepsy in patients outside the Old Order Amish community and demonstrates an association between *CNTNAP2* and schizophrenia. Our results highlight *CNTNAP2* as a strong candidate gene for epilepsy and schizophrenia. The results further support the notion that CNV is a potentially important source of genetic susceptibility to both complex brain diseases.

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