

Common variation in *NCAN*, a risk factor for bipolar disorder and schizophrenia, influences local cortical folding in schizophrenia

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Background. Recent studies have provided strong evidence that variation in the gene neurocan (*NCAN*, rs1064395) is a common risk factor for bipolar disorder (BD) and schizophrenia. However, the possible relevance of *NCAN* variation to disease mechanisms in the human brain has not yet been explored. Thus, to identify a putative pathomechanism, we tested whether the risk allele has an influence on cortical thickness and folding in a well-characterized sample of patients with schizophrenia and healthy controls.

Method. Sixty-three patients and 65 controls underwent T1-weighted magnetic resonance imaging (MRI) and were genotyped for the single nucleotide polymorphism (SNP) rs1064395. Folding and thickness were analysed on a node-by-node basis using a surface-based approach (FreeSurfer).

Results. In patients, *NCAN* risk status (defined by AA and AG carriers) was found to be associated with higher folding in the right lateral occipital region and at a trend level for the left dorsolateral prefrontal cortex. Controls did not show any association ($p > 0.05$). For cortical thickness, there was no significant effect in either patients or controls.

Conclusions. This study is the first to describe an effect of the *NCAN* risk variant on brain structure. Our data show that the *NCAN* risk allele influences cortical folding in the occipital and prefrontal cortex, which may establish disease susceptibility during neurodevelopment. The findings suggest that *NCAN* is involved in visual processing and top-down cognitive functioning. Both major cognitive processes are known to be disturbed in schizophrenia. Moreover, our study reveals new evidence for a specific genetic influence on local cortical folding in schizophrenia.

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Introduction

Schizophrenia is a severe mental illness that affects 1% of the population worldwide. Among the major psychiatric disorders, schizophrenia shows one of the highest estimates of heritability, ranging between 60%

and 90% (Nöthen *et al.* 2010). However, the neurobiological abnormalities determined by this genetic risk are poorly understood. In a recent genome-wide association study (GWAS) and in a follow-up study (Cichon *et al.* 2011), we found strong statistical evidence that the single nucleotide polymorphism (SNP) rs1064395 in the 3' untranslated region of the neurocan (*NCAN*) gene on chromosome 19p13.11 is a risk factor for bipolar disorder (BD). In view of formal genetic and molecular genetic evidence that BD and schizophrenia share some of their risk factors, we performed a subsequent association study and identified that this

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common variation is also a risk factor for schizophrenia (Mühleisen *et al.* 2012). Both studies found that the A allele of rs1064395 is the risk-mediating allele. These results suggest that the SNP rs1064395 influences risk to a broader psychosis phenotype. In the next step, imaging studies are necessary to test the hypothesis that the *NCAN* risk allele mediates pathophysiologically relevant changes in the brain.

NCAN, formerly termed *CSPG3*, encodes neurocan, a brain-specific chondroitin sulfate proteoglycan (CSPG) of the lectican family (Prange *et al.* 1998). Recent analyses of mRNA expression patterns in mice and humans suggest an important role for *NCAN* in the developing mammalian brain (Cichon *et al.* 2011; see also BrainSpan: Atlas of the Developing Human Brain; www.brainspan.org). Neurocan molecules are mainly expressed by reactive astrocytes, but also by neurons (Karetko & Skangiel-Kramska, 2009). Neurocan interacts with cell surface molecules, such as neural cell adhesion molecule (NCAM), L1/neuronglia (L1/Ng)-CAM, transient axonal glycoprotein-1 (TAG-1)/axonin-1 and an N-cadherin-binding N-acetylgalactosaminyl phosphoryltransferase (GalNAcPTase) (Rauch *et al.* 2001). These features enable neurocan to participate in the modulation of cell adhesion and migration. Furthermore, neurocan and other lecticans are thought of as 'organizers' of the extracellular matrix (ECM) in the central nervous system (Yamaguchi, 2000). From embryogenesis to early postnatal stages, neurocan is one of the predominant lecticans in the brain ECM whereas, later in life, other members of this protein family prevail (Frischknecht & Seidenbecher, 2008; Karetko & Skangiel-Kramska, 2009). Characterization of *NCAN* double-knockout mice demonstrated impaired pre-pulse inhibition (Miró *et al.* 2012), an intermediate phenotype for schizophrenia and also for BD (Thaker, 2008). The affected mice also showed higher exploratory and overall locomotor activity. Accordingly, in patients with schizophrenia and BD, the risk allele was strongly associated with the symptom dimension overactivity, providing further support for an association between *NCAN* and psychosis pathophysiology (Miró *et al.* 2012). Despite these strong molecular and behavioural data, the possible relevance of *NCAN* variation for disease mechanisms in the human brain has not yet been explored.

In the past few years, imaging genetics has emerged as a promising approach in neuropsychiatric research. It enables identification and characterization of genetic variants that influence brain structure and function underlying normal and abnormal biological pathways (Esslinger *et al.* 2009; Erk *et al.* 2010; Schultz *et al.* 2011; Stein *et al.* 2012). In the present study, we investigated the SNP rs1064395 for a potential effect on cortical morphology using a clinically well-characterized

sample of 63 patients with a DSM-IV diagnosis of schizophrenia and 65 healthy controls, all of German origin. We measured two parameters for cortical shape (cortical thickness and cortical folding) and subdivided the patients and controls according to their *NCAN* risk status. Cortical thickness is assumed to approximate the distance between the pial surface (outer surface) and the border between the grey and white matter (inner surface) of the cortex. As major parts of the formation of gyri (and sulci) take place in the third prenatal trimester (White & Hilgetag, 2011), cortical folding can be regarded as a sensitive marker for aberrations in neurodevelopment. Alterations in cortical thickness (Kuperberg *et al.* 2003; Narr *et al.* 2005a,b; Fornito *et al.* 2008; Nesvåg *et al.* 2008; Schultz *et al.* 2010b,c) and cortical folding (McIntosh *et al.* 2009; Schultz *et al.* 2010a; Palaniyappan *et al.* 2011) have been associated repeatedly with schizophrenia. Additionally, both structural measures are highly heritable (Rogers *et al.* 2010; Winkler *et al.* 2010) and are therefore suitable intermediate phenotypes for our approach.

Carrying the minor allele A of rs1064395 has been shown to confer a risk effect that increases disease susceptibility and, conversely, carrying the G-allele can be assumed to establish a protective effect (Cichon *et al.* 2011; Mühleisen *et al.* 2012). We therefore hypothesized that carriers of the genotypes AA and AG exhibit aberrant cortical thickness and/or cortical folding in comparison to carriers of the genotype GG.

Method

Subjects

We studied 63 patients with schizophrenia and 65 controls. All participants were right-handed (Annett, 1967). Diagnoses based on the SCID were established by a clinical psychiatrist (I.N.) and confirmed by two independent psychiatrists (R.G.M.S. and C.C.S.). All patients met DSM-IV criteria for schizophrenia and had no second psychiatric diagnosis. Of the 63 patients, 58 were diagnosed as paranoid, two as undifferentiated, two as disorganized and one as catatonic subtype of schizophrenia according to DSM-IV. They were on stable medication, mostly with second-generation antipsychotics.

Healthy controls were screened for major medical, neurological and psychiatric history. None of the controls had a current psychiatric disorder, or a history of psychiatric disorder, and none had first-degree relatives with a psychiatric disorder according to DSM-IV. Exclusion criteria for all participants were neurological disease or damage, or medical disorders potentially influencing neurocognitive function. Ancestry was assigned to patients and controls on the basis of

Table 1. Demographic and clinical data

	Genetic groups			<i>p</i>
	GG	AA/AG		
Healthy controls				
<i>n</i>	51	14		
Age (years), mean (s.d.)	26.7 (6.8)	25.6 (8.1)		0.593
Patients				
<i>n</i>	49	14		
Age (years), mean (s.d.)	29.4 (10.0)	26.9 (10.0)		0.410
PANSS total, mean (s.d.)	69.2 (26.5)	73.9 (28.7)		0.565
PANSS positive, mean (s.d.)	15.8 (7.3)	19.3 (8.3)		0.150
PANSS negative, mean (s.d.)	18.1 (6.9)	17.4 (10.2)		0.788
CPZE, mean (s.d.)	479.8 (419.1)	283.3 (208.1)		0.122

PANSS, Positive and Negative Syndrome Scale (Kay *et al.* 1987); CPZE, chlorpromazine equivalents; s.d., standard deviation.

Participants were grouped according to their NCAN risk status, that is homozygous and heterozygous risk genotype (AA+AG) *versus* the homozygous non-risk genotype (GG) of the single nucleotide polymorphism (SNP) rs1064395. There were no significant differences with regard to age and gender between the resulting genotype groups (*p* values > 0.190).

p values resulting from a two-sample *t* test.

self-reported ancestry. All participants were of German origin. None of the subjects were consanguineous. All participants gave written informed consent to the study, which was approved by the Ethics Committee of Friedrich-Schiller University. Sociodemographic and psychopathological data are given in Table 1.

Magnetic resonance imaging (MRI)

MRI acquisition

We acquired high-resolution anatomical T1-weighted MRI scans in a 1.5-T Siemens Magnetom Vision whole-body system (Erlangen, Germany) using a three-dimensional spoiled gradient echo sequence: 1-mm-thick sagittal slices with repetition time (TR)=15 ms, echo time (TE)=5 ms, flip angle=30°, field of view (FOV)=256 mm × 256 mm, matrix=256 × 256, number of sagittal slices=192. All scans were inspected for motion artefacts and a neuroradiologist confirmed the absence of gross pathological findings.

MR scan processing and calculation of cortical shape measures

We used the FreeSurfer software package (version 4.0.5; <http://surfer.nmr.mgh.harvard.edu>; Dale *et al.*

1999; Fischl *et al.* 1999) for image processing and for thickness and folding analyses. Image processing comprised the removal of non-brain tissue, transformation to Talairach-like space and segmentation of grey/white matter tissue. The white/grey matter boundary was tessellated and topological defects were corrected automatically. After intensity normalization, transition of the grey/white matter and pial boundary was identified by detecting the greatest shift in intensity through surface deformation. The entire cortex of each participant was then inspected visually and any inaccuracies in segmentation were edited manually.

Computation of cortical thickness

Cortical thickness was computed by finding the shortest distance between a given point (node of vertices) on the estimated pial surface and the grey/white matter boundary and *vice versa* and averaging these two values (Fischl & Dale, 2000). As the cortical maps generated were not restricted to the voxel resolution of the original data, they were capable of detecting sub-millimetre differences between diagnostic groups. This automated approach of cortical thickness measurement has been validated against manual measurements in schizophrenia (Kuperberg *et al.* 2003).

Computation of cortical folding

Absolute mean curvature represents a very reliable local measure for cortical folding alterations, allowing a fine-grained comparison of cortical folding covering the entire cortex (Gaser *et al.* 2006; Luders *et al.* 2006). Absolute mean curvature of the pial surface was calculated as described by Schultz *et al.* (2010a) for approximately 150 000 nodes in each hemisphere of the brain.

Genotyping and determination of subgroups according to the risk status

For the patients, rs1064395 genotypes were extracted from a genome-wide data set that had been genotyped and quality controlled in the study by Rietschel *et al.* (2011). For the controls, lymphocyte DNA was isolated from venous blood samples using standard methods. To target SNP rs1064395 a primer pair was designed. Amplicons were generated using standard polymerase chain reaction (PCR) conditions. These were then sequenced using the Sanger method and a 3130XL Genetic Analyzer (Applied Biosystems, USA). Electropherograms were analysed by two independent investigators using SeqMan II (DNASTar, USA).

Depending on the NCAN risk status, patients and controls were divided into subgroups resulting in

Table 2. Results of the cluster analysis for cortical folding in patients with schizophrenia

Cortex area	Size (mm ²)	Talairach coordinates			CWP	<i>p</i> value (nominal)
		x	y	z		
Right lateral occipital	623.1	25.3	−95.0	3.6	0.032	1.00×10 ^{−4}
Left dorsolateral prefrontal	526.4	−7.1	24.4	52.9	0.073	6.10×10 ^{−5}

CWP, Cluster-wise probability.

CWP (corrected for multiple testing) resulting from the cluster analysis in each cortical hemisphere; nominal (uncorrected) *p* values resulting from Student's *t* tests comparing the average cortical folding values at the identified anatomical clusters (as illustrated in Fig. 1).

two genotype groups for each diagnostic group, that is 2×(AA+AG) and 2×GG, for statistical analyses. We compared risk allele (A) carriers with non-risk allele carriers in patients and controls separately, that is controls: AA+AG versus GG; patients: AA+AG versus GG. There were no significant differences with regard to age and gender between the resulting genotype groups (*p* values>0.190).

Statistical analyses

Statistical cortical maps

Cortical thickness and cortical folding measurements at each vertex of the subjects' surface were mapped onto a common spherical coordinate system using a spherical transformation. Maps were smoothed using a Gaussian kernel of 10 mm. A general linear model (GLM) controlling for the effect of age was used to estimate differences in cortical thickness and cortical folding at each node. The GLM is based on an ANCOVA approach. Right and left hemispheres were tested separately.

Monte Carlo simulation and clustering

For correction of multiple comparisons, a Monte Carlo simulation (10000 iterations) and a cluster analysis were performed to identify contiguous clusters of significant cortical thickness and cortical folding differences between the two genotype groups for each analysis (*p*<0.05). The simulation and clustering approach is based on the AlphaSim algorithm (Ward, 2000).

Results

Effect of NCAN risk status on the two cortical shape measures

Schizophrenia patients carrying the homozygous and heterozygous risk genotypes (AA, AG) exhibited

significantly higher cortical folding covering parts of the right visual cortex (V1 and V2 areas) than patients with the GG genotype (CWP=0.032; Table 2, Fig. 1 b). A statistical trend (CWP=0.073) was found for the left dorsolateral prefrontal cortical folding in the schizophrenia risk carriers (Table 2; Fig. 1a). Extraction of the cortical folding values from these clusters demonstrated comparable highly significant nominal *p* values: *p*=1×10^{−4} for the lateral occipital cluster and *p*=6.10×10^{−5} for the prefrontal cluster.

No effect of NCAN risk status on cortical folding was observed in the controls. For cortical thickness, no effect of the risk variant was found, either in the patients or in the controls.

Genotype×diagnosis interaction

To test for differing effects of the rs1064395 genotype on diagnosis, we additionally performed a genotype×diagnosis interaction analysis as in prior imaging genetics studies (Prata et al. 2009), using the same statistical parameters as defined for the initial analyses (Monte Carlo simulation, clustering). We found a significant genotype×diagnosis interaction for cortical folding in the right superior parietal cortex, that is the influence of the NCAN variant on cortical folding was significantly stronger in patients (in the direction of a higher cortical folding in risk carriers) than in controls.

Comparison of cortical thickness and folding between patients and controls

We analysed the main effect of diagnosis on cortical thickness and folding *post hoc* using the same statistical conditions as defined for the primary analyses. Patients showed significant cortical thinning in several cortical areas including the left superior, middle and inferior temporal cortex, left inferior parietal, right inferior frontal, orbitofrontal, right superior, middle and inferior temporal cortex, in line with previous

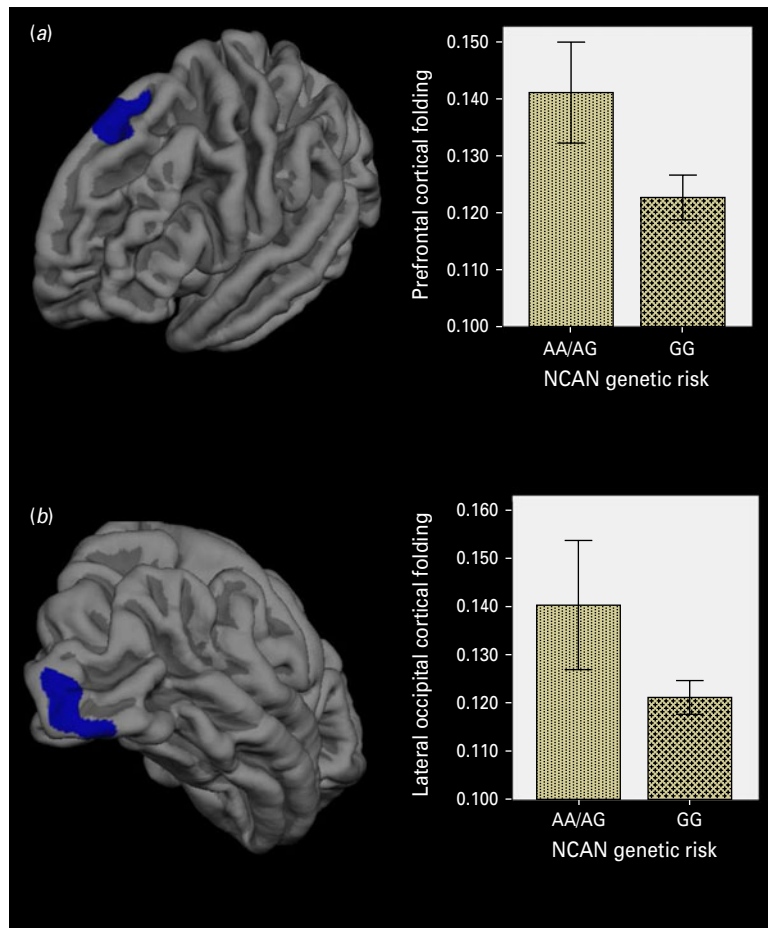


Fig. 1. Effect of the neocan (NCAN) risk status on cortical folding in patients with schizophrenia. Statistical maps show the identified anatomical clusters (coloured in blue) encompassing regions in (a) the left prefrontal and (b) the right lateral occipital cortex. Bar diagrams illustrate the folding differences between risk allele carriers (AA+AG) compared to non-risk allele carriers (GG) of the single nucleotide polymorphism (SNP) rs1064395. Mean cortical folding values extracted from the anatomical clusters between risk and non-risk carriers. Error bars indicate 95% confidence interval.

findings (Kuperberg *et al.* 2003; Narr *et al.* 2005a,b; Nesvåg *et al.* 2008; Schultz *et al.* 2010b,c). We found no significant cortical folding differences between patients and controls.

In a subsequent analysis, we compared cortical thickness and folding only in risk allele carriers using the same statistical parameters as defined for the initial analysis (entire cortex analysis, Monte Carlo Simulation and automated clustering). In patients, we found a significant cortical thinning with the AA/AG genotype compared to controls with the same genotype covering smaller parts of the left inferior and middle temporal region and the right inferior temporal, fusiform and lateral occipital cortex. No significant effects were detected for cortical folding.

Discussion

The investigated NCAN variant is one of the few genetic risk factors for major psychoses (BD, schizo-

phrenia) that has received genome-wide support and robust evidence for replication in follow-up studies. To the best of our knowledge, the present study is the first to characterize the impact of this variant on the cortex structure in humans.

Effect of NCAN variation on occipital and frontal cortical folding

Our main finding was that cortical folding in association with NCAN risk status was significantly altered in parts of the primary and secondary visual areas (V1, V2) of the right occipital lobe in patients with schizophrenia, but not in the matched controls. Both V1 and V2 are related to early visual processing and deficits for these areas have been reported for schizophrenia (Butler & Javitt, 2005). Disturbed cortical integration of visual and other sensory inputs is thought to be involved in cognitive disruption in schizophrenia, as suggested by current bottom-up models (Javitt,

2009). Although the visual cortex in schizophrenia seems to be relatively free from reductions in grey matter volume (Honea *et al.* 2005), several independent studies of schizophrenia have found alterations of occipital cortical folding (White *et al.* 2003; Narr *et al.* 2004; Schultz *et al.* 2010a, 2013). Hence, the observed association between the *NCAN* risk variant and disturbed occipital cortex folding suggests a role for *NCAN* in visual processing. Future studies are warranted to validate and further explore this potential function of *NCAN*.

Second, we found a strong statistical trend in the left hemisphere for altered dorsolateral prefrontal cortical folding in patients with *NCAN* risk. Prefrontal dysfunction is thought to be a major constituent of altered cognitive control in schizophrenia (Minzenberg *et al.* 2009), with crucial functions in higher-level gating and top-down processing (Miller, 2000). Thus, the joint finding of fronto-occipital cortical folding alterations provides indirect support for an integrative model of both higher-order and early sensory processing deficits in the cortex of schizophrenia patients (Silbersweig & Stern, 1996; Adcock *et al.* 2009; Hugdhal, 2009; Hahn *et al.* 2010; Neuhaus *et al.* 2011; Waters *et al.* 2012). Moreover, our findings might reflect a potential pathomechanism for an additive disruption of top-down and bottom-up cortical processing associated with schizophrenia.

Third, we found a significantly stronger effect of the genotype on right superior parietal cortical folding in patients compared to controls in the direction of higher cortical folding in risk carriers. Hence, this finding supports the observed effects of the *NCAN* risk genotype on cortical folding in patients. The superior parietal cortex is known as a crucial structure for the reorganization of information during working memory processing (Koenigs *et al.* 2009). Disturbed working memory is one of the major pathophysiological features in schizophrenia (Forbes *et al.* 2009). Both prefrontal and superior parietal dysfunction constitute important elements in the context of disturbed cognitive control and associated altered working memory deficits in schizophrenia (Minzenberg *et al.* 2009). Thus, our finding of a significant genotype \times diagnosis interaction strengthens the view that *NCAN* might be involved in altered higher-order cognitive functioning in schizophrenia.

In view of our previous result that *NCAN* variation is also a common risk factor for BD (Cichon *et al.* 2011), it is of note that top-down cognitive dysfunction (Hill *et al.* 2008; Reichenberg *et al.* 2009) and visual processing deficits (Green *et al.* 1994; Yeap *et al.* 2009) are thought to be neurobiological features of both major psychoses. This observation might lead to the conclusion that specific common cognitive features in schizophrenia and BD are based on overlapping

genetic risk variants such as *NCAN*. In view of the recent aetiological discussions about the psychosis spectrum, we are of the opinion that only a convergent integration of genetic, cognitive and neuroimaging features can shed further light on this important issue.

Cortical folding as an intermediate phenotype associated with NCAN variation in schizophrenia

An important aspect of our study is that the *NCAN* risk variant demonstrated an association with cortical folding but not with cortical thickness. This observation provides further support for accumulating evidence that specific neuroimaging features can be phenotypically and genetically independent (Panizzon *et al.* 2009; Winkler *et al.* 2010). Our data suggest that *NCAN* might be specifically related to cortical folding rather than cortical thickness. This provides the first genetic evidence for a specific underpinning of localized cortical folding aberrations in schizophrenia.

As suggested by the spatiotemporal gene expression patterns in mice and humans (Cichon *et al.* 2011), along with the functions of *NCAN* known to date (Frischknecht & Seidenbecher, 2008; Karetko & Skangiel-Kramska, 2009; Miró *et al.* 2012), the common genetic variant in *NCAN* most probably exerts its disease-relevant effect on cortex structure when major folding processes (gyrification) take place (White *et al.* 2003). After the cortical folding pattern has been established, it is largely maintained during life (Armstrong *et al.* 1991). In line with this, mechanical models of brain development in primates indicate that folding is driven by the formation of cortico-cortical connections (Hilgetag & Barbas, 2006), and further animal experiments have demonstrated that early disruption of white matter connections are related to focal hypergyria (Goldman-Rakic, 1980). This view is also supported by a recent study by Dauvermann *et al.* (2012), who analysed prefrontal gyrification together with functional connectivity. Overall, our results, in conjunction with prior knowledge, indicate that increased prefrontal gyrification might be related to increased short-range and reduced long-range connectivity. Thus, our finding that genetic variation in *NCAN* is specifically associated with altered cortical folding, a neurodevelopmental cortical marker, fits well with previous molecular and neuroimaging studies.

Based on current knowledge, the question of how *NCAN* (encoding neurocan) contributes precisely to these neurodevelopmental processes at the molecular level cannot be answered definitively. In humans, *NCAN* is expressed in many cortical and subcortical regions (see BrainSpan: Atlas of the Developing Human Brain; www.brainspan.org). The expression

in the cortex peaks during development starts to fall after birth, and decreases continually until the end of life (Human Brain Transcriptome (transcriptome data and associated metadata for the developing and adult human brain, www.hbatlas.org); Johnson *et al.* 2009; Kang *et al.* 2011). Therefore, high NCAN expression levels coincide with the maturation of folding and connectivity patterns. This, and its molecular properties as an ECM molecule, enable neurocan to participate in the modulation of cell adhesion and migration processes during corticogenesis. As cortical folding, in addition to the mechanical properties of axonal fibres ('tension-based hypothesis'), is thought to be caused by synergistic effects of cell proliferation and differentiation ('grey matter hypothesis') (Zilles *et al.* 2013), it seems likely that NCAN proteins are involved in cortical folding. The common genetic variant (SNP rs1064395) that we have analysed in the present study is a risk factor for schizophrenia and also for BD, but has not yet been characterized at the molecular genetic level. Our imaging genetics study provides the first evidence for a potential effect of the risk variant (A allele) on cortex structure. How the variant or another variant in linkage disequilibrium contributes exactly to an aberrant folding in the occipital and frontal regions remains to be elucidated in further follow-up studies.

Limitations

Cortical folding is regarded as an important anatomical feature in schizophrenia (Mangin *et al.* 2010). In the present study we demonstrated the influence of NCAN on frontal and occipital cortical folding using an entire cortex approach. However, it cannot be excluded that other regions might be affected using region-of-interest (ROI) approaches. Thus, further studies with independent samples are needed to explore the role of NCAN in disease architecture.

Regarding the comparison of cortical folding between patients and controls, it might be of relevance that the numbers of risk allele carriers in both diagnostic groups were equal. This might explain why we could not detect any folding differences between the diagnostic groups as we demonstrated that the NCAN SNP influences cortical folding in patients.

One further limitation is that, because of the lack of longitudinal data, we cannot provide information about differences with regard to psychosis outcome, which might be influenced by the risk variant in association with the influence on cortical folding. Thus, longitudinal studies on this issue are warranted.

Conclusions

This is the first study to describe a potential neuro-anatomical pathomechanism driven by a NCAN risk

variant in schizophrenia patients. In particular, we used a systematic imaging genetics approach across both hemispheres and identified that the risk allele or alleles in linkage disequilibrium with it influence the folding of the occipital and prefrontal lobe in patients but not in controls. This finding indicates a potential role for NCAN in higher-order cognitive functioning and early visual processing. Both major cognitive processes are known to be disturbed in schizophrenia. Our study also provides evidence for a specific genetic underpinning of localized cortical folding aberrations in schizophrenia. This work is a good starting point for future studies, which should further investigate the suggested functional aspects along with the possible role of NCAN variation in brains of patients with BD. In general, we conclude that it might be beneficial to assess cortical folding as an additional intermediate phenotype in future studies of the cortex structure.

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Declaration of Interest

None.

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