Proteomic, genomic and translational approaches identify CRMP1 for a role in schizophrenia and its underlying traits

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Schizophrenia is a chronic illness of heterogenous biological origin. We hypothesized that, similar to chronic progressive brain conditions, persistent functional disturbances of neurons would result in disturbed proteostasis in the brains of schizophrenia patients, leading to increased abundance of specific misfolded, insoluble proteins. Identification of such proteins would facilitate the elucidation of molecular processes underlying these devastating conditions. We therefore generated antibodies against pooled insoluble proteome of post-mortem brains from schizophrenia patients in order to identify unique, disease-specific epitopes. We successfully identified such an epitope to be present on collapsin-response mediator protein 1 (CRMP1) in biochemically purified, insoluble brain fractions. A genetic association analysis for the CRMP1 gene in a large Finnish population cohort (n = 4651) corroborated the association of physical and social anhedonia with the CRMP1 locus in a DISC1 (Disrupted-in-schizophrenia 1)-dependent manner. Physical and social anhedonia are heritable traits, present as chronic, negative symptoms of schizophrenia and severe major depression, thus constituting serious vulnerability factors for mental disease. Strikingly, lymphoblastoid cell lines derived from schizophrenia patients mirrored aberrant CRMP1 immunoreactivity by showing an increase of CRMP1 expression, suggesting its potential role as a blood-based diagnostic marker. CRMP1 is a novel candidate protein for schizophrenia traits at the intersection of the reelin and DISC1 pathways that directly and functionally interacts with DISC1. We demonstrate the impact of an interdisciplinary approach where the identification of a disease-associated epitope in post-mortem brains, powered by a genetic association study, is rapidly translated into a potential blood-based diagnostic marker.

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INTRODUCTION

The neurobiology of chronic mental illnesses such as schizophrenia or the recurrent affective disorders is still largely uncharted territory. One obstacle to progress has been the lack of biological correlates for the so far purely clinical diagnoses of these diseases. There is an emerging understanding that the mere phenotypical diagnosis of chronic mental illnesses is flawed and should rather be determined via phenomes (1) or endophenotypes (2). Endophenotypes are defined as reductionist physiological, biochemical or psychological variables in patients, but also healthy relatives of patients, indicating a vulnerability to chronic mental illness. Heritability of psychosis-prone traits or endophenotypes is well established (3–6) and the identification of genes associated with these phenotypes provides valuable information towards ultimately dissecting the biological mechanisms of mental diseases. The usefulness of identifying clinical endophenotypes is demonstrated by the ability to model some of them in experimental conditions with animal models such as prepulse inhibition (7) or anhedonia (8).

The phenotypical, clinical diagnosis of schizophrenia is obtained by clinical interview and self-reporting of the patient according to internationally defined criteria like those of Diagnostic and Statistical Manual of Mental Disorders—Fourth Edition (DSM IV) (9) and International Classification of Diseases 10 (ICD-10). Positive (hallucinations, delusions), negative (affective flattening, social withdrawal) and cognitive (attention, verbal memory) symptoms characterize the clinical picture of schizophrenia. The term ‘schizophrenia’ comprises very different clinical courses (10,11) and this heterogeneity likely reflects a situation where different biological causes converge into a similar, final common behavioral phenotype. It is therefore likely that different sub-groups within the clinical diagnosis schizophrenia can be described by specific endophenotype signatures. The difficulties in aligning current clinical diagnoses with underlying biology is highlighted, for example, by the fact that many chronic mental illness-related genes are associated with or linked to clinical phenotypes which span current diagnostic boundaries (12,13). For that reason, endophenotypic traits are not expected to be restricted to just one chronic mental illness, but may equally cross current diagnostic boundaries.

Anhedonia, i.e. the inability to experience pleasure from activities usually found enjoyable, has long been recognized not only as a key endophenotype of major depressive disorder and a chronic, negative symptom of schizophrenia, but also as a risk factor for future development of these diseases, reflecting a vulnerability trait (14). In behavioral neuroscience, anhedonia is considered a reward-related behavioral deficit and as such can be clearly linked to neuronal circuitry involved in anticipation, decision-making and avolition in animal models (8). The DISC1 (Disrupted-in-schizophrenia 1) gene, a prime candidate gene linked (15) and associated (16,17) with schizophrenia and other chronic mental illnesses has been genetically associated with anhedonia in an unselected birth cohort in Finland (18). In addition, several animal models of DISC1-related brain disease display increased immobility in the forced swim test (19–21), which is seen as a correlate of anhedonia (22), suggesting that the DISC1 gene is contributing to the human endophenotype anhedonia, and as such is a vulnerability trait for chronic mental illness itself (23,24). Of note, genetic evidence from the original Scottish pedigree (15) implicating a role for DISC1 in chronic mental illnesses has been stronger than analysis of common variants (25), similar to that which has been seen with genes related to Alzheimer’s disease (AD), where amyloid precursor protein (APP) and the presenilins are established rare Mendelian risk factors but fail to produce any evidence in studies of the non-Mendelian forms of AD (26). AD research has demonstrated that the study of such rare genes has been pivotal to understanding disease pathogenesis (26), and a similar situation may also apply for DISC1 and DISC1-interacting factors.

We were interested in identifying novel biological genes related to endophenotypes of chronic, non-acute symptoms of chronic mental illnesses such as anhedonia under the assumption that the identification of such genes would lead to the identification of key molecular processes involved in the biology of the chronic progressive underlying processes in schizophrenia.

Even though there is no obvious, unambiguous neuropathology in schizophrenia (27), it is clear that chronic symptoms in schizophrenia are progressive and irreversible in the absence of effective treatments, and that in most cases they include a relative decline in intellectual abilities from premorbid levels (10,11,28,29). In the absence of detectable cell death in schizophrenic brains, neuronal dysfunction may instead involve less clearly identifiable forms of aberrant activity or inactivity.

Proteostasis defines a cell’s ability to maintain homeostasis of correctly folded proteins during any cellular imbalance caused by cell stress, spontaneous misfolding or failing of subsequent chaperoning or degradation. Proteostasis in post-mitotic neurons is sensitive to prolonged functional disturbances and its disequilibrium results in aggregated or insoluble proteins (30). We reasoned that dysfunctional neurons arising as a result of chronic functional disturbance in schizophrenia might present with a proteostatic imbalance with the disease-specific accumulation of insoluble proteins, similar to that which has previously been shown for the mental illness candidate protein DISC1 (31). The DISC1 gene is thought to occupy a key position in the network of genes or proteins that control behavior (23,24). DISC1 expression is functionally regulated by other mental disease genes such as neuregulin 1 (32) and is functionally involved in the reelin disabled-1 pathway (33). DISC1 aggregates show exclusive characteristics not present in their soluble counterparts by recruiting other, independently identified mental disease candidate proteins like dysbindin (34), and have been demonstrated to impair axonal transport in primary neurons (35).

Here, we purified the insoluble proteome from a pool of schizophrenic post-mortem brains and identified a unique epitope present only in the schizophrenia, but not the normal brain control pool. This epitope was identified as being present on collapsin response mediator protein 1 (CRMP1). We demonstrate that the CRMP1 gene is associated with the endophenotype of anhedonia in a DISC1-dependent manner, and that a CRMP1 epitope has high diagnostic potential to discriminate schizophrenia-derived lymphoblasts from those of normal controls. Epitope discovery powered by genetic association and peripheral epitope quantitation is an important identification strategy in translational psychiatry.
RESULTS

Disease-associated epitope discovery

To identify subtle brain pathology, we purified the insoluble proteome, or ‘aggregome’, from BA23 tissue from a pool (n = 15) of schizophrenia patients from the Stanley Medical Research Institute’s Consortium Collection (SMRI CC, Baltimore, MD, USA) (31,36) and used aliquots thereof to immunize mice for monoclonal antibody (mAB) generation by standard techniques (37,38). The idea was to evoke a specific and highly sensitive humoral immune response directed to misfolded conformers of proteins and thus to have the intact immune system select epitopes of misfolded proteins similar to which has been demonstrated for multimeric αβ in AD (39) or oligomeric α-synuclein in Parkinson’s disease (40). Our hypothesis was that schizophrenia was most likely to contain such misfolded proteins since, among all chronic mental illnesses, it is the disease with the most debilitating course.

The resulting hybridomas were selected for their ability to recognize pooled schizophrenia brain homogenates over pooled normal control brain homogenates (Fig. 1A). From the immunization of one mouse, we obtained approximately 1300 hybridoma clones whose supernatants were screened by a standardized dot blot method, the enzyme-linked immunonitration assay (38,41) and found that one antibody recognized a clear linear epitope that was highly enriched in the schizophrenia brain homogenate pool (see also Supplementary Material, Table S1). Using a protein array (RZPD, Berlin, Germany; 42), we identified the collapsin response mediator protein 1 (CRMP1) as harboring an epitope recognized by this antibody (Supplementary Material, Fig. S1).

The disease-associated epitope discovery approach identified CRMP1 as a possible candidate protein for schizophrenia-related traits in post-mortem brain homogenates. The identified mAB #A clearly reacted with recombinant GST-CRMP1 and mouse CRMP1 (Fig. 1B and C). For subsequent experiments, we switched to a commercial antibody raised against a linear epitope in the middle portion of the CRMP1 protein (#3625, ProSci, CA, USA) due to its higher sensitivity and to avoid possible artifacts resulting from transferring antibodies with a potential conformation-specific or polyspecific component (43) to assays where linearized epitopes are abundant.

Higher immunoreactivity of the disease-associated aggregated versus normal control could be validated when single-aggregome samples of the SMRI CC, constituting the original immunogen pool, were blotted and probed using an independent, commercial α-CRMP1-specific antibody (ProSci #3625; Fig. 1D and E). The same aggregome samples were also investigated using the original mAB #A, yielding an almost complete overlap in immunoreactivity (Supplementary Material, Fig. S2); the slight differences may be due to variation in the position of the epitope recognized on CRMP1, or conformation-specific components within mAB #A (43).

Of note, normalization of the total protein content prior to quantitation of aggregated CRMP1 was done based on the protein concentration within starting material using the very same mass of starting material before purification (protein content in 10% w/v brain homogenates) such that the immunoreactive CRMP1 in the blotted pellet (Fig. 1D) reflected the relative differences in insoluble CRMP1 in between each of the simultaneously processed brain samples. To control for differential basal protein expression between samples that could influence aggregation propensity, quantitative measurements of insoluble CRMP1 were corrected by the expression level of CRMP1 in the homogenate (Fig. 1E). Samples of all chronic mental illness cases were pooled since it is increasingly recognized that biological factors or genotypes cross clinical phenotypes (12,13,44). The highest amount of insoluble CRMP1 was found in schizophrenia and bipolar disorder brains (Supplementary Material, Fig. S3). For some samples, degradation of insoluble CRMP1 was visible by a smear of lower molecular weight CRMP1 immunoreactive bands (Fig. 1D). Positive cases were not correlated with any particular race or clinical condition (i.e. psychosis at the time of death) or any other variable (substance abuse, post-mortem interval, lifetime drug dosage, disease duration, etc.) for which data were available from the SMRI CC.

Aggregation propensity of CRMP1 isoforms

To investigate whether CRMP1 had an aggregation propensity of its own or whether it was co-precipitating through interaction with another aggregated protein, we over-expressed untagged CRMP1sv in human neuroblastoma cells. CRMP1 has been described to exist in two splice forms, short variant (sv) and long variant (lv) (45). CRMP1sv corresponds to the major species detected in human brain (Fig. 1B) and can be cleaved by calpain to a shorter fragment (46); these fragments were also observed in our data (Fig. 1D, Supplementary Material, Fig. S4A). We performed a biochemical insolubility assay similar to that performed with the post-mortem brain homogenates; we did not observe a major proportion of CRMP1sv in the insoluble pellet (Fig. 2A) and the protein regularly dispersed within the cell (Fig. 3A). We therefore hypothesized that the presence of CRMP1 in the purified insoluble fraction of post-mortem brains could be due to co-aggregation with another genuinely aggregating protein, in a manner similar to the recruitment of soluble dysbindin to insoluble DISC1, which we have described previously (34). Indeed, co-expression of CRMP1sv with DISC1 led to a co-aggregation in the insoluble pellet of DISC1 aggresomes, indicating that CRMP1sv was pulled to the pellet in a DISC1-dependent manner (Fig. 2A). This could be further confirmed by immunostaining of NLf cells co-expressing an enhanced green fluorescent (eGFP)-CRMP1 fusion protein and an mRFP-DISC1 fusion protein, where we saw recruitment of formerly soluble eGFP-CRMP1 to mRFP-DISC1 aggresomes (Fig. 2B). Direct interaction between recombinant GST-CRMP1sv and a fragment of human His6-tagged DISC1 (corresponding to amino acids 316–854) (47) generated in Escherichia coli could be demonstrated in a cell-free pulldown assay (Fig. 2B). Interestingly, the non-abundant CRMP1lv isoform that had been reported to have antagonistic functions to CRMP1sv in cell migration (48) had an aggregation propensity of its own (Supplementary Material, Fig. S4). DISC1 and CRMP1 could also be shown to co-localize in the cytosol of cortical neurons from the human brain (Fig. 2C, Supplementary Material, Figs S5 and S6), demonstrating that the interaction of endogenous CRMP1 and DISC1 is also possible. However, the absence of a strong overlap of aggregated
DISC1 with aggregated CRMP1 (Supplementary Material, Fig. S2) suggests that other factors or proteins may also play a role in CRMP1 aggregation. Within a proteomic study that will be published separately (Ramos et al., manuscript in preparation), we identified a regulatory interaction between DISC1 and CRMP1, with CRMP1 expression being increased in DISC1-silenced primary mouse neurons CRMP1 (Fig. 2C), a finding which could partially explain the lack of overlap between aggregated CRMP1 and DISC1 in post-mortem brains (Supplementary Material, Fig. S2) even though interaction is possible in vitro (Fig. 2A and B).

Genetic association of CRMP1 with endophenotypes of the schizophrenia continuum

We further explored the CRMP1 gene for genetic association to chronic mental illness-related endophenotypes, mining existing data from the large Northern Finland birth cohort 1966 (NFBC66, n = 4651) with quantitative measures of psychosis proneness (49). Two SNPs (rs3821936 and rs3755849) displayed experiment-wide significant association with measures of social and physical anhedonia. With the minor alleles of both SNPs, increasing scores (indicating worsening phenotypes) were found on both measures with similar effect sizes (Supplementary Material, Tables S2 and S3, Fig. S7). Interestingly, these SNPs only associated when a previously identified DISC1 association was taken into account through conditioning of the sample. rs3821936 displayed significant association (P = 0.0027) with physical anhedonia in individuals who carry a protective DISC1 variant, whereas rs3755849 displayed significant association (P = 0.0025) with social anhedonia when the entire DISC1 risk model was used as a covariate—this being predominantly driven by individuals carrying DISC1 risk variants.
Supplementary Material, Tables S2 and S3, Fig. S7). This demonstrated a degree of genetic interplay between CRMP1 and DISC1 alongside the protein interaction. Further, both SNPs are located within the same LD haplotype block that covers exon 6 to exon 12 of the CRMP1 gene (Supplementary Material, Fig. S8), with these two SNPs defining distinct haplotypic backgrounds. Thus, the genetic factor at CRMP1 that increases risk for chronic mental illness is likely dependent upon the genetic background at DISC1.

CRMP1 identifies schizophrenic patients in blood-derived material

To investigate whether the presence of a CRMP1 epitope was mirrored in a more easily accessible tissue from patients with chronic mental illness, in particular in schizophrenia patients where anhedonia is an integral part of the chronic negative symptoms, we isolated lymphocytes from schizophrenic patients (n = 15) and normal controls (n = 15; Fig. 4A), and
immortalized them with Epstein Barr virus (EBV). The resulting lymphoblastoid cells from patients diagnosed with schizophrenia showed a clear elevation of CRMP1 immunoreactivity upon lysis (Fig. 4A and C), whereas, for comparison, DISC1 expression levels were not associated with clinical diagnosis (Supplementary Material, Fig. S9). CRMP1 positivity was not correlated to race, or any scales available for psychotic or negative symptoms (Supplementary Material, Table S5; calculations performed with Pearson’s correlation test, two-tailed). The increase in CRMP1 immunoreactivity was paralleled by an increase in CRMP1 mRNA (Supplementary Material, Fig. S10), indicating that the increase in protein expression in lymphoblastoid cells is at least to a large part due to an increase in gene transcription. We also probed a limited collection of lymphoblasts obtained from patients suffering from bipolar disorder and found positive CRMP1 immunoreactivity only in two out of seven patients (#5/#6; Fig. 4B). Furthermore, we confirmed significant elevation of CRMP1 immunoreactivity in a completely independent sample of lymphoblasts from patients diagnosed with schizophrenia compared with those derived from controls (Fig. 4D).

This difference in CRMP1 immunoreactivity was only obvious in proliferating lymphoblasts, as arresting of lymphoblastoid cell growth with retinoic acid led to CRMP1 expression leveling out between the schizophrenia and control groups (data not shown). To our knowledge, such clear differences in the level of immunoreactivity of an epitope have so far not been reported on a protein in lymphoblasts derived from schizophrenia patients versus controls. The distinct and specific epitope of CRMP1 is thus mirrored in patient-derived lymphoblasts and offers a potential window for biological diagnosis of chronic mental illness.
In this paper, we establish that disease-specific epitope discovery during disturbed proteostasis of dysfunctional neurons is an efficient method for the identification of chronic mental illness-related proteins and genes, and that endophenotype-associated epitopes in the brain can be mirrored in blood-derived lymphoblasts as well as in the corresponding genes. The method we present thus allows us to unify genetic and post-mortem brain analyses with biological diagnosis. Even though each of the methods of analysis is entirely independent, they converge on a consistent picture linking CRMP1 to a major endophenotype of chronic mental illness.

We propose that the method of epitope discovery here presented for a CRMP1 epitope may also lead to the discovery of yet different disease-associated epitopes in similar experiments with post-mortem brains from other diseases.

It is important to realize that many antibodies isolated from naturally occurring antibodies are actually conformation-specific or polyspecific in their molecular recognition (43,50) rather than recognizing a single linear epitope. The isolation of the latter often represents a rather artificial selection for laboratory purposes or the result from immunizing with an immunogenic peptide. It is therefore advantageous to switch after initial epitope discovery identifying misfolded protein candidates to more reliable antibodies optimized for

**DISCUSSION**

Figure 4. (A) Representative western blot of whole lymphoblastoid cell lysates from patients with schizophrenia (S) or normal controls (C) probed with α-CRMP1 antibody (#3625, ProSci), α-DISC1 antibody 14F2 (34) and α-GAPDH antibody as loading control (lower panel), as indicated. (B) Western blot of whole lymphoblastoid lysates from patients with bipolar disorder probed with α-CRMP1 antibody (#3625, ProSci). The western blot identifies cases #5 and #6 as positive for CRMP1 immunoreactivity. (C) Quantitative densitometry of α-CRMP1 immunoreactivity of lymphoblasts from schizophrenic patients (n = 14) and normal controls (n = 15), normalized by immunoreactivity to GAPDH [samples comprise those in (A)]. Lymphoblasts from patients with schizophrenia displayed significantly higher CRMP1 immunoreactivity. ***Mann–Whitney U test (no Gaussian distribution), two-tailed P < 0.001. (D) Quantitative densitometry of α-CRMP1 (#3625, ProSci) of an entirely independent collection of lymphoblastoid cell lines (University of Düsseldorf, Germany) from those in (A) and (C) (Johns Hopkins Medical School, USA), demonstrating significant, disease-associated differences in CRMP1 immunoreactivity (see Supplementary Material, Table S6 for demographic information).
standard laboratory procedures to be used in follow-up experiments on their biochemistry and cell biology.

Within this epitope discovery paradigm, the possibility that antibodies are elicited by proteins present in only a few patients is, in our view, not in contradiction to a general applicability of the method since we do expect biological heterogeneity within the clinical disease category of chronic mental illnesses. To include as many potential subgroups of patients as possible, a pool of a large number of patients is desirable. The presence of the epitope in the brain of only a few patients (Fig. 1D) or in the lymphoblasts of a subgroup of patients (Fig. 4A) is therefore consistent with the notion that schizophrenia is biologically heterogenous. To what extent the presence of a selective CRMP1 epitope is a defining signature for one particular subgroup of schizophrenia or other chronic mental illnesses will have to be confirmed in a larger group of cases.

CRMP1 is a protein that has been identified to mediate semaphorin-3a-dependent signaling of neurons in the developing brain (51) and is involved in reelin-dependent radial neuronal migration (52). It heteromerizes with CRMP2, encoded for by a gene that has previously been linked to schizophrenia and other brain diseases by regulating glutamate signaling through the WAVE1/Rac1 complex (reviewed in 53). CRMP1 possesses roles in cytoskeleton maintenance by stabilizing microtubule assembly (54) and has splice-form-dependent functions in F-actin polymerization (48), a process critically regulated during synapse formation, maintenance or neuronal migration. CRMP1lv was identified to be regulated by cdc42 (55), a protein activated by reelin (56) and regulated by NDEL1, which is an important downstream target of DISC1 (57). Thus, CRMP1 lies at the intersection of both the DISC1 and reelin pathways (Fig. 5), providing further evidence for the convergence of schizophrenia-associated disease pathways (34,58,59).

We previously demonstrated that DISC1 aggresomes can recruit otherwise freely dispersed and soluble proteins such as dysbindin (34). For CRMP1sv, we have here demonstrated a similar mechanism of interaction with aggregated DISC1 (Fig. 2A). The protein interaction between CRMP1sv and aggregated DISC1 (Fig. 2A and B) was complemented by a genetic interaction between DISC1 and CRMP1 (Supplementary Material, Table S2), with CRMP1 association with the anhedonia endophenotypes being dependent on an allele of DISC1 previously associated with anhedonia and CRMP1 (18). Our studies now suggest that the CRMP1 gene adds to this risk.

Specifically, the minor allele of rs3821936 increases (worsens) scores of anhedonia, with such an effect being observed regardless of DISC1 genetic modifiers although greater in those individuals with a DISC1 neutral genetic status. Further, scores on the anhedonia scales rapidly increase in heterozygotes for rs3821936 in combination with DISC1 neutral status, but do not increase in combination with DISC1 protective status. In contrast, the minor allele of rs3755849 increases scores on the anhedonia scales dependent on the presence of DISC1 genetic modifying factors, only having an effect in individuals either with DISC1 risk or protective status, but not in individuals of a DISC1 neutral status. It should be kept in mind that although the CRMP1 variants increase scores equally in the DISC1 risk and protective status groups, the DISC1 protective groups still have overall lower scores for the anhedonia scales (Supplementary Material, Fig. S7). At this point, the precise molecular conditions under which endogenous CRMP1 segregates to the insoluble fraction and how insoluble CRMP1 disrupts brain circuitry ultimately leading to behavioral endophenotypes remain to be determined.

The identification of peripheral, easily accessible diagnostic markers for chronic mental illness has been a long-sought goal.
in these still entirely clinically diagnosed illnesses and it is our hope that the method we present here may lead to the identification of biological signatures of chronic mental illness subtypes. In two independent sets of lymphoblastoid cells, CRMP1 immunoreactivity was clearly increased in schizophrenic patients compared with normal controls (Fig. 4C and D), indicating that lymphoblasts can be used as a window into the brain regarding CRMP1-related molecular pathology. The intriguing question arises of whether the same molecular factors that lead to insoluble CRMP1 in postmitotic neurons may also lead to an over-expression in proliferating lymphoblasts, thus explaining this seemingly unlikely and counter-intuitive connection. It cannot be excluded that the same route of cellular pathology involved in chronic mental illness-related endophenotypes takes different shapes according to its cellular context: CRMP1 aggregation in the brain and its relative overexpansion in proliferating tissue. CRMP1 is functionally linked to the reelin pathway [(52); see Fig. 5], a key signaling molecule in the developing brain, and in this context it is notable that reelin transcripts have also been identified in plasma cells (60) even though for a long time reelin has been seen as a protein whose functions and presence are confined to the CNS. Reelin abnormalities have long been associated with disorders of the schizophrenia continuum or behavioral control (61,62).

To summarize, disease-associated epitope discovery powered by genetics is a novel, fast and efficient way to identify molecules relevant in chronic brain conditions like chronic mental illness starting from protein pathology and providing direct links to genetics and ultimately aid in the identification of disease-specific biological markers for use in diagnosis.

**MATERIALS AND METHODS**

**Aggregome purification**

Brodman Area 23 (BA23) frozen cortex (posterior cingulum) was obtained from the SMRI CC, by availability. Posterior cingulum has been consistently designated to be an anatomical region of interest in chronic mental illness (63) (see ref. 36 and http://www.stanleyresearch.org for more extensive information on demographics). The SMRI CC (60 cases) consists mainly of Caucasian race, with three Asian and two African-American samples. Sarkosyl-insoluble fractions from human brain samples and transfected neuroblastoma cells were obtained as described in Leliveld et al. (31).

**Antibody generation and screening**

Aggregome fractions from post-mortem brains with the diagnosis schizophrenia were pooled (n = 15) and used for immunization of mice for obtaining monoclonal antibodies by fusion of splenocytes with myeloma cells according to standard procedures as described before (38). Resulting hybridoma supernatants were screened by a standardized dot blot procedure as described (38) in a semi-quantitative manner for increased signal intensity of the brain homogenate pool of schizophrenia brains (BA23; n = 15) versus normal brains (BA23; n = 15) from the SMRI.

**Protein array**

For epitope determination, a protein array containing expressed sequence tags and full-length clones of a random, human cDNA library generated from fetal brain was used. The high-density protein macroarray (library name: hEx1eDNA_sub, library No: 800) was obtained from the Deutsches Ressourcenzzentrum für Genomforschung Ressourcenzyt (RZPD), which is now Imagenes, Germany (www.imagenes.de) (42). Bioinformatic analysis and clone identification was performed using the RZPD homepage online tool. Filters were analyzed in a manner similar to Western blots, with the exception that TBS-T (50 mM NaCl, 120 mM Tris–HCl, pH 7.5, 0.05% Tween 20, 0.5% Triton X-100) was used for washing steps. Secondary antibody only was used as negative control.

**Pulldown experiments**

For pulldown of recombinant GST-CRMP1 with recombinant His$_{6}$-tagged DISC1(316–854) generated in E. coli (47), 4 mg of recombinant His$_{6}$-tagged DISC1(316–854) were coupled to 1 ml of activated NHS-Sepharose (Amersham, USA) overnight at 4°C and blocked for 2 h with 100 mM Tris, pH 8.0. For each reaction, 50 μl beads were incubated with 10 μg recombinant GST-CRMP1 or controls in PBS at 4°C overnight. Next, beads were washed three times with PBS and resuspended in loading buffer containing 5% β-mercaptoethanol. Following SDS–polyacrylamide gel electrophoresis (PAGE) and blotting, membranes were incubated with α-CRMP1 antibody (ProSci) at a concentration of 1:1000.

**Cell labeling studies**

eGFP-CRMP1sv/-CRMP1lv, red fluorescent (mRFP)-full length DISC1, non-tagged full-length DISC1 and non-tagged CRMP1sv/CRMP1lv were cloned into pcDNA3.1 (+), then transfected into CAD mouse neuroblastoma cells or human NLF neuroblastoma cells (Children’s Hospital of Philadelphia, Philadelphia, PA, USA) with metafectene (Biontex, Germany) at 70% confluency. For microscopy, cells were grown on glass cover slips. Fixation in 4% paraformaldehyde/PBS, pH 7.4, was performed 24 or 48 h after transfection, and, if necessary, immunostaining for intracellular proteins was carried out as described (64). All images were taken on a Zeiss LSM 510 confocal microscope or an Axio Imager M2 (Apotome2; Zeiss, Germany).

**DISC1 knock down in primary neurons**

DISC1 was silenced in E14–15 murine primary neurons by means of shRNAs using lentiviral particles (65). Cell lysates were subjected to 2D electrophoresis: the first dimension was on immobilized pH gradient (IPG) strips 4–7, 24 cm (GE Healthcare); the second dimension was by SDS–PAGE on 10% gels. Following electrophoresis, gels were fixed in 10% methanol/7% acetic acid for 1 h, and stained overnight with Sypro Ruby fluorescent dye (Bio-Rad, USA). Gels were subsequently washed for 1 h in 10% methanol/7% acetic acid, and scanned in a Pharos FX Plus (Bio-Rad).
Image analysis was performed with the Ludesi REDFIN 3 software. The integrated intensity of each of the spots was measured, and the background corrected and normalized. Differential expression of proteins was defined on the basis of ≥2-fold change between group averages and \( P < 0.05 \). Protein spots chosen for mass spectrometric analysis were excised from the gels and manually in-gel-digested with trypsin. Mass spectrometric data were obtained in an automated analysis loop using 4800 MALDI-TOF/TOF analyzer (Applied Biosystems).

Antibodies

Rabbit α-CRMP1 antibody was obtained from ProSci (Poway; #3625). In addition, a monoclonal α-CRMP1 antibody 4C12 was generated to peptide VEGAYENKTIDFDAYS corresponding to residues 39–54 of human CRMP1, according to standard procedures after immunizing a mouse with KLH-linked peptides for four times (41). Generation of human DISC1-specific mAB 14F2 has been described (34).

As secondary antibodies, anti-rabbit-Alexa Fluor 488, anti-mouse-Alexa Fluor 594 or anti-mouse-FITC (all from Invitrogen, Germany) were used for cellular stainings. An anti-rabbit-POD from Pierce (USA) for western blotting. GAPDH antibody was from Santa Cruz (USA, #sc-137179). Western blots were analyzed by densitometry using the ImageJ software (NIH, USA).

Immunohistochemistry

Formalin-fixed section of the cortex (BA9) from a normal individual (received from the SMRI) was deparaffinized, blocked for endogenous peroxidase and pretreated with target retrieval solution (Dako, Germany, #S1699). After blocking with Dako #S2022, primary α-DISC1 antibody 14F2 was applied at 1 μg/ml overnight, then incubated with the first secondary antibody Histofine (Histofine, Germany; #414131F) anti-mouse POD for 30 min; diaminobenzidine (DAB; Vector SK-410, Vector, Germany) was used as substrate. For staining with the second antibody, after the inactivation of peroxidase with hydrogen peroxide (0.3%), rabbit α-CRMP1 (ProSci #3625) was applied at 2 μg/ml for 2 h at room temperature. Subsequently, again secondary antibody Histofine (414131F) anti-mouse POD was incubated for 60 min and HistoGreen substrate (Linaris, Germany, #E109) applied.

Genetic association

The genetic analysis mined pre-existing data from studies of NFBC66. These data are fully described in the previous work with this cohort from which this study is derived (18,49). This study has followed the Declaration of Helsinki and its amendments in full. The study has been approved by the Ministry of Social Affairs (Finland) and the Ethical Committee of The Northern Ostrobothnia Hospital District. All the participants in the study have provided their written informed consent. In short, from an original cohort of 12 058 live births in Northern Finland during 1966, 4651 individuals have completed a psychometric questionnaire at age 31, provided DNA and given written informed consent. The psychometric scales that were used included the Revised Social Anhedonia Scale (SAS) (L. Chapman, unpublished test, 1982) (66), Revised Physical Anhedonia Scale (PHAS) (L. Chapman, unpublished test, 1978) (66) and Schizoidia Scale by Golden and Meehl (67). These scales were selected to assess normal properties that correspond to positive and negative aspects of psychotic disorders, while also being valid indicators of later development of psychosis (18).

To study the CRMP1 gene, SNPs that have been genotyped as part of the GWAS study using the Illumina Infinium Human CNV370-Duo marker set (total number of SNPs = 339 054) were identified from a region 10 kb upstream to 10 kb downstream (chr4: 5 854 804–5 965 211; UCSC hg18 build). A total of 28 SNPs were identified and their \( P \)-values for the three endophenotypes were extracted from the data from previous studies. This included the results from a routine GWAS analysis of the three endophenotypes, in addition to results from analyses of SAS and PHAS conditioned on DISC1 risk modulating variants. The DISC1 variants used for conditioning were combinations of three SNPs, rs1538979, rs821577 and rs821633, which have previously been observed to associate in the NFBC66 cohort with both SAS and PHAS (18). This association is through a model of interplay between the three SNPs, first identified in a study of schizophrenia and bipolar disorder (62) and replicated under hypothesis in the NFBC66 cohort (18). None of these SNPs had provided results of genome-wide significance in the previous studies. Association analysis had been performed using PLINK (version 1.05) (68), and its additive linear regression model, in order to determine the presence of significant differences in group scores for these quantitative measures, where the groups are defined by genotype.

In order to distinguish between significant association and false discovery at the candidate gene level, multiple testing correction was performed. Since the multiple SNPs and multiple phenotype models (total number of tests = 308) are, to some degree, related, the Bonferroni correction method is considered overly conservative. To account for LD between markers, we used the SNP spectral decomposition method (69) with modifications by Li et al. (70) to determine the effective number of independent marker loci (Meff) (\( n = 19.2162 \)) and the Meff-adjusted experiment-wide significance threshold required to keep type I error rate at 5% (\( P \leq 0.0027 \)).

In order to study how the pattern of associations relate to each other and the CRMP1 gene, haplotype blocks of LD were determined using the HapMap CEU data according to the method previously described (71,72). We identified eight haplotype blocks, with our most significant findings highlighting the same haplotype block that spans from intron 6 to intron 12 of the CRMP1 gene.

Lymphoblasts

Lymphocytes were obtained from peripheral blood of patients with schizophrenia and control subjects at Johns Hopkins University Medical School (Fig. 4A and B) and were infected with Epstein–Barr virus, followed by incubation at 37°C for 20–25 days as described previously to yield immortalized lymphoblasts (73,74). A second set of lymphoblasts was obtained from the Department of Psychiatry at the Heinrich Heine
University of Düsseldorf (Fig. 4D) and similarly immortalized by EBV infection according to standard protocols (75). All patients were diagnosed according to the DSM-IV criteria (9). All subjects were administered the Structured Clinical Interview for DSM-IV Axis I Disorders-Clinician Version and all patients were assessed with the Scales for the Assessment of Positive and Negative Symptoms by study psychiatrists. Study psychiatrists also assessed the subjects’ capacity to give informed consent and our study was approved by IRB at Johns Hopkins University (Study #NA_00037204). IRB from Howard University (10-MED-40) or by a positive vote from the Ethics Committee of the Medical Faculty of the University of Düsseldorf (studies #1790/2702). For some experiments, lymphoblastoids growing in suspension were treated for 7 days with 1 μM retinoic acid in order to arrest growth, with 50% of the cell media being siphoned off at 3 and 5 days after the start of treatment and being replaced with fresh retinoic acid-containing media.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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