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Neuropeptide precursor VGF is genetically associated with social anhedonia and underrepresented in the brain of major mental illness: its downregulation by DISC1

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In a large Scottish pedigree, disruption of the gene coding for DISC1 clearly segregates with major depression, schizophrenia and related mental conditions. Thus, study of DISC1 may provide a clue to understand the biology of major mental illness. A neuropeptide precursor VGF has potent antidepressant effects and has been reportedly associated with bipolar disorder. Here we show that DISC1 knockdown leads to a reduction of VGF, in neurons. VGF is also downregulated in the cortices from sporadic cases with major mental disease. A positive correlation of VGF single-nucleotide polymorphisms (SNPs) with social anhedonia was also observed. We now propose that VGF participates in a common pathophysiology of major mental disease.

INTRODUCTION

A neuropeptide precursor VGF is a neuroprotective protein that is known to regulate neuronal energy metabolism has an important role in neural processes such as adult neurogenesis, synaptic plasticity and also harbors an antidepressant-like activity (1–5). Expression of VGF has been found to be decreased in leukocytes of patients from major depression and in brains of

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patients affected with bipolar disorder (6,7). With regard to schizophrenia, data are conflicting: increased concentration of a VGF-derived peptide has been reported in the cerebrospinal fluid (CSF) of patients with schizophrenia (8); however, results from the Stanley Medical Research Institute (SMRI) Genomics Database showed decreased VGF in prefrontal cortices of schizophrenic patients (6). More recently, reduced density of hypothalamic VGF-immunoreactive neurons was detected in brains from patients with schizophrenia as compared with controls (9).

In a large Scottish pedigree, disruption of the gene coding for DISC1 clearly segregates with major depression, schizophrenia and related mental conditions (10). Hence, DISC1 might be used as a 'molecular Rosetta stone' to explore the molecular mechanisms underlying mental disease (11), just as rare mutations in the amyloid precursor protein (APP) and presenilin genes were used to decipher the molecular underpinnings of Alzheimer's disease (12). DISC1 is an intracellular scaffold protein and mediates multiple roles in neurodevelopment and neurosignaling by interacting with many molecules, such as Glycogen synthase kinase-3β (GSK3β), nuclear distribution protein nudE-like 1 (NDEL1), TRAF2 and NCK-interacting protein kinase (TNIK), Kalirin and cAMP-specific 3′,5′-cyclic phosphodiesterase 4B (PDE4B) (11,13). In the Scottish pedigree, loss of DISC1 function (e.g. dominant-negative and/or haploinsufficiency) is likely to underlie the pathophysiology of the mental conditions (14,15). Likewise, many preclinical studies have supported the views that loss of DISC1 function leads to biological and circuitry changes relevant to major mental illness (11).

Therefore, in the present study, we initially explored molecular changes elicited by loss of DISC1 function by utilizing a proteomic approach: loss of DISC1 leads to a reduction in the expression of VGF. We also report that VGF is downregulated in the brains from patients with major mental illness and is genetically associated with social anhedonia.

RESULTS

Proteomic screening in cells with DISC1 knockdown: a marked reduction of VGF

We performed an unbiased proteomic study aimed at identifying proteins directly affected by loss of DISC1 function. SH-SY5Y cells were initially chosen as a convenient neuronal model. DISC1 was knocked down by using lentiviral particles carrying five different shRNAs (Supplementary Material, Fig. S1 and Table S1). The five shRNA sequences used exhibited different efficacies: silenced 1 exhibited the highest efficacy, with only 4.3% of DISC1 residual expression, as calculated by real time-PCR (Supplementary Material, Fig. S1B and Table S2). Proteomic analysis of control and DISC1-knocked down SH-SY5Y cells was carried out by using four gels for each condition, analyzed with Redfin Ludesi 3 software (16). Several spots with fold intensity change ≥ 2 and $P < 0.05$ were detected (to be reported elsewhere), and were excised and subjected to MALDI analysis after in-gel tryptic digestion. Among these, 14 different spots, most of them located in two different groups with molecular weights (MWs) ∼90 and 80 kDa, were found to correspond to VGF, and were all downregulated with a fold change between 2 and 11.5 (Fig. 1, Supplementary Material, Fig. S2 and Table S3). Considering their similar MW but different isoelectric points, they might result from different post-translational modifications, including limited cleavage. To date, VGF has been detected by western blot analysis as a doublet of 80–90 kDa, even though its predicted molecular weight is 68 kDa and no post-translational modifications have yet been identified (17). Since recombinant VGF also migrates at this unexpectedly high position, the behavior has been attributed to the high content of proline residues in VGF (17). Several short peptides derived from VGF, such as AQEE30, TLQP-21, TLQP-62, Neuroendocrine Regulatory Peptide-1 (NERP-1) or Neuroendocrine Regulatory Peptide-2 (NERP-2) have been described (18). To our knowledge, however, this is the first time that VGF variants with apparent MWs close to that of full-length VGF are reported. We are currently investigating whether these correspond to post-translational modifications and/or limited proteolytic trimming of full-length VGF.

DISC1 knockdown leads to a reduction of VGF expression in primary neurons

Encouraged by the results obtained with SH-SY5Y cells, we prepared murine primary neurons (combined cortical and hippocampal neurons). DISC1 was knocked down by using three different shRNAs, introduced through lentiviral particles (Fig. 2A). The DISC1 RNAi #1 construct was chosen for these experiments because it has been used previously (19), resulting in 85% DISC1 downregulation (Fig. 2B and C). Non-specific effects of the infection on DISC1 expression were ruled out by lack of differences between untreated cells and cells treated with viral particles carrying control sequences (Supplementary Material, Fig. S3).

Knockdown of DISC1 led to a ∼75% drop in the levels of VGF (Fig. 2B and D), a result that was reproducibly obtained with all three constructs used to silence DISC1 (Fig. 2A), in agreement with results obtained with SH-SY5Y cells. The very substantial downregulation of VGF produced in all the DISC1 silencing conditions suggests that these results are not due to a specific effect produced in a particular cell line.

To validate further the specificity of DISC1-induced regulation of VGF expression, a conditional stable cell line where full-length non-mutant human DISC1 was inducibly overexpressed (by addition of 1 mg/ml doxycycline) was generated. VGF was upregulated in this line with respect to control cells (Supplementary Material, Fig. S4).

We also examined changes of molecules associated with DISC1 signaling, when VGF downregulation occurred in response to DISC1 knockdown (Supplementary Material, Fig. S5). We observed downregulation of the expression of phosphorylated AKT and CREB, while the expression of phosphoERK and Brain derived neurotrophic factor (BDNF) remained unchanged in the DISC1 knocked down cells.

Reduction of VGF in brains from patients with major mental illnesses

Decreased levels of VGF mRNA have been previously described in hippocampus and prefrontal cortex regions of human bipolar postmortem brains (6). Given that loss of DISC1 function is associated with a wide range of major mental illness, including schizophrenia, major depression and bipolar disorder (14), we

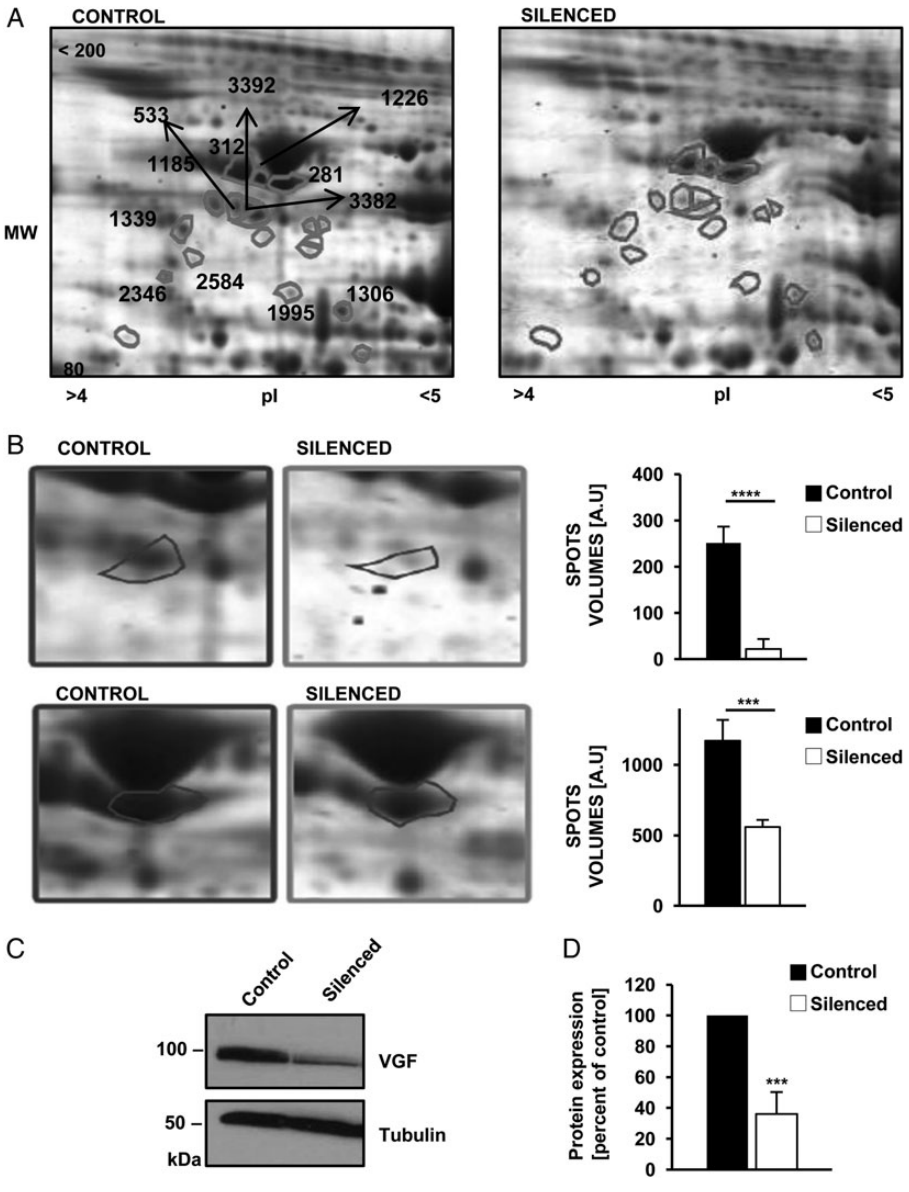

Figure 1. VGF expression in SH-SY5Y cells. (**A**) 2D gel areas containing most spots corresponding to VGF in representative DISC1 silenced (right) and control (left) 2D-PAGE gels. (**B**) Volumetric quantification of two representative spots, spot 3382, with a fold-negative change of 11.52, $P < 0.0001$, and spot 281, with a fold change of 2.09, $P < 0.001$. (**C**) VGF western blot analysis of control and silenced cells. (**D**) Quantification of data presented in C ($n = 6$ independent experiments). Total protein was normalized to tubulin. Error bars represent SD. Statistical analysis was carried out by the (paired) two-tailed Student's *t*-test. $^{***}P < 0.001$, $^{****}P < 0.001$.

measured and compared levels of VGF in cortical tissue of a group of patients with these disorders compared with those from normal controls (20). Lower expression of VGF was found in the group of psychiatric patients (Fig. 3). These lower levels of VGF expression did not correlate with occurrence of psychosis, substance abuse, duration of disease, medication or tissue variables such as pH or postmortem interval (Supplementary Material, Table S4). Only a gender correlation was observed and females showed significantly decreased VGF levels, independent of illness or diagnosis (Supplementary Material, Table S4 and Fig. S6).

In the same set of samples, we did not detect differences in DISC1 expression between patient and control groups (Supplementary Material, Fig. S7). In these samples we failed to find correlation between VGF levels and the previously reported aggregation of DISC1 in a subset of cases with mental disease (21) (Supplementary Material, Fig. S8). These results indicated that DISC1 does not contribute as a major driver to the changes in VGF levels in the sporadic cases in the brain set.

Genetic association of the *VGF* gene with social anhedonia

Through mining of the existing genetic data we were able to test for the presence of genetic variation at the VGF genomic locus that associates with chronic mental illness-related endophenotypes. To control for the effective number of tests performed

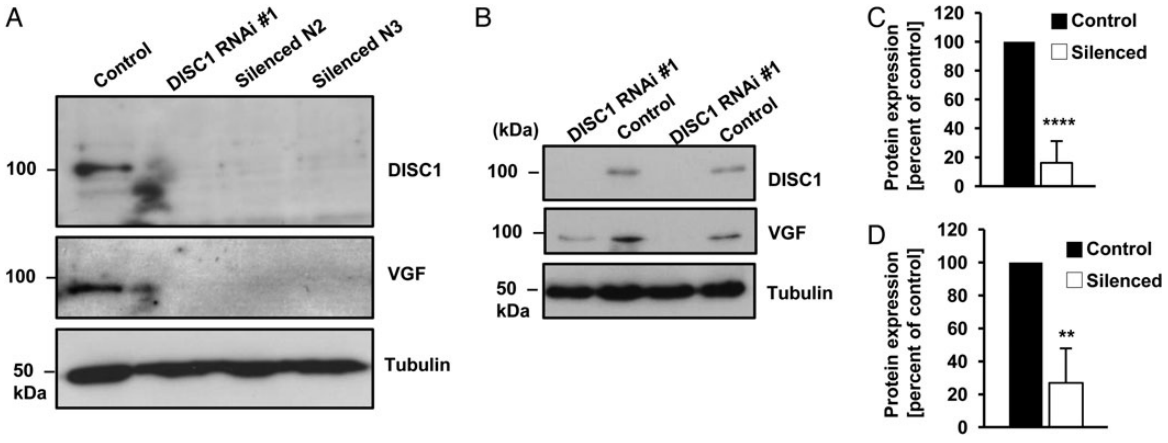

Figure 2. DISC1 and VGF expression in primary neuron cell cultures. (**A**) DISC1 and VGF expression in neuron cells infected with three different shRNA constructs to knock-down DISC1. A non-target shRNA was used as control. (**B**) Expression of DISC1and VGF in cells infected with lentiviral particles containing the construct 'DISC1 RNAi #1'. (**C**) Quantification of DISC1 knockdown shown in (B). (**D**) Quantification of VGF expression shown in B. (C, D): $n = 8$ independent experiments. Error bars represent SD. Statistical analysis was carried out by the (paired) two-tailed Student's *t*-test. Data were normalized to tubulin. $^{**}P < 0.01$, $^{****}P < 0.0001$.

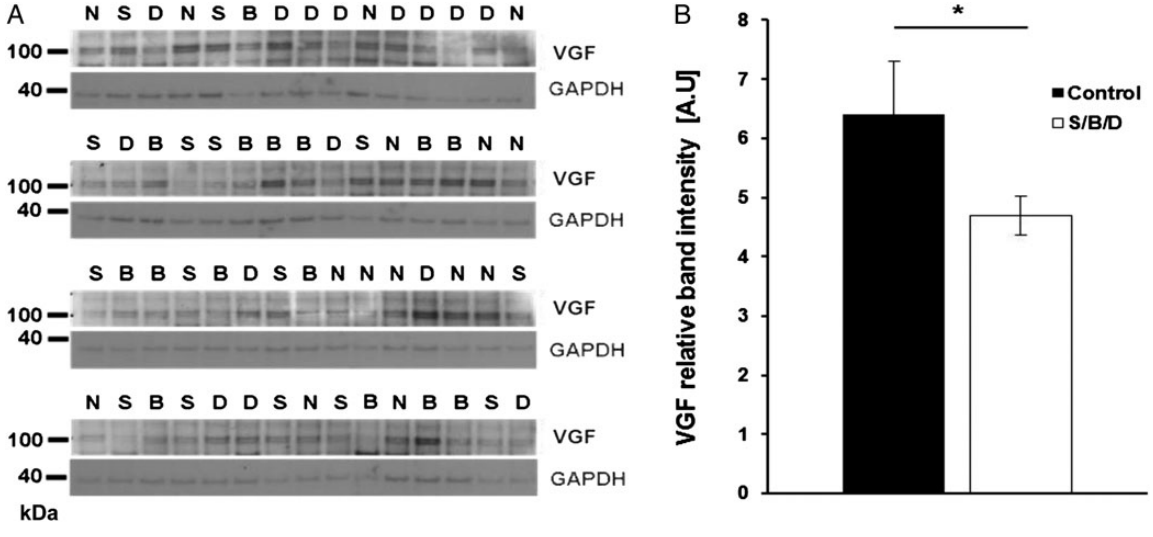

Figure 3. Expression of VGF in brains of patients with mental disease. (**A**) Western blot analysis of the SMRI samples [normal controls (N), patients with bipolar disorder (B), major depression (D) and schizophrenia (S)] (**B**) Densitometric analysis of the normalized VGF signal of SMRI collection brains. VGF signal intensity is decreased in patients with psychiatric illnesses ($n = 44$) compared with normal controls ($n = 15$); error bars represent SEM. Statistical analysis was carried out by using unpaired one-tailed Mann–Whitney *U*-test; $P = 0.031$) (B: bipolar disorder/D: major depression/ S: schizophrenic patients)

($n = 15$) through this data mining an experiment-wide threshold of $P = 0.0033$ was set. Two SNPs, out of the four in the region, were thus observed to associate significantly with the social anhedonia scale (rs1048303 $P = 0.0025$, beta $= -0.385$; rs734688 $P = 0.0019$, beta $= -0.408$) (Fig. 4). Analysis of linkage disequilibrium (LD) over the VGF region demonstrates that both SNPs could be indicative of the same haplotypic background (Supplementary Material, Fig. S9); however, the two significant SNPs are not within strong LD with each other, $r^2 = 0.59$ (Supplementary Material, Table S5). No significant association was observed for either physical anhedonia (PHAS) or golden and meehl schizodia scale (GM).

In the model including DISC1 as a covariate the observed significance increased with social anhedonia (SAS) (Supplementary Material, Table S6), with the breakdown of individuals depending on their DISC1 status establishing that VGF plays a significant role in the variability of these traits when in combination with risk-modifying factors at the DISC1 locus, this role was further echoed through the observation of significant association between PHAS and rs1048303 only in those individuals carrying DISC1 risk increasing alleles (Supplementary Material, Fig. S10 and Table S6). No interaction effect between VGF and DISC1 was found, demonstrating an additive, rather than epistatic, effect between these variants (Supplementary Material, Table S7).

DISCUSSION

The main finding of this study is that the neuroprotective VGF protein is downregulated in the brains from sporadic cases of

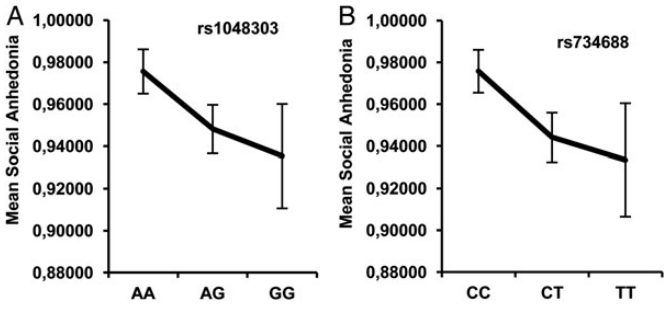

Figure 4. Average performance on the social anhedonia scale for: (**A**) each genotype of SNP rs1048303; (**B**) each genotype of SNP rs734688. Graphs show the mean scores for the logarithmically transformed scales of the Revised Social Anhedonia Scale. 95% Confidence intervals are shown.

major mental illness. Reduction of VGF was also observed upon DISC1 knockdown. We also observed that VGF is genetically associated with social anhedonia, an endophenotype that underlies a wide range of major mental illness.

Diagnostic and Statistical Manual of Mental Disorders or International Statistical Classification of Diseases (ICD)-based diagnostic classification is based on good reliability and clinical utility, whereas biological validity is not fully considered by definition. As a result, it is reasonable that no clear-cut correlation of one molecular trait with one single diagnosis is seen. Likewise, in the Scottish pedigree, the same mutation (disruption of *DISC1* gene) leads to mental conditions labeled with different diagnoses (such as schizophrenia, bipolar disorder and major depression) in the family members. This perspective matches the efforts in the Research Domain Criteria from the NIH, which focuses on specific domains (e.g. cognition and motivation) associated with specific neural circuitry (e.g. that involving the prefrontal cortex) and molecular markers, instead of focusing on a specific disease diagnosis. In the present study, reduction of VGF may be associated with social anhedonia, which underlies a wide range of major mental illness, including schizophrenia, bipolar disorder and major depression.

We used DISC1 as an entry point or an initial lead or driver for research to elucidate an important cascade that might be relevant to the pathophysiology of mental conditions. As a result of DISC1 knockdown we found a reduction of VGF expression. The reduction of VGF was also observed in the brains of 'sporadic' cases with major mental illness. Thus, it is reasonable to conceive that multiple types of etiologies may contribute to 'common' downstream pathophysiology (that is, in this case, a reduction of VGF). Very mild or even negligible contribution of DISC1 to the VGF change in the brain samples is consistent with this notion. There is precedence that depicts a similar relationship of multiple etiologies and common downstream pathophysiology in mental condition (22): a mouse model representing a condition of human 22q11 deletion frequently associated with major mental illness displays disturbance of CXCR4/CXCL12 signaling and interneuron deficits. In olfactory neuronal cells from 'sporadic' cases with schizophrenia, a reduction of CXCL12 is observed without any change in the key driver in the 22q11 locus. Therefore, the present study supports the working hypothesis, also defended in other studies, that although the etiologies of mental disease are diverse, they may converge in some common pathophysiologies.

MATERIALS AND METHODS

Cell culture

SH-SY5Y (European Collection of Cell Cultures, Salisbury, UK) were maintained in 1:1 Earle's balanced salt solution-F12HAM (Sigma-Aldrich, Germany) with 15% fetal bovine serum (FBS) (Gibco, Life Technologies, Gaithersburg, MD, USA), 1% Glutamine (Gln) (Sigma-Aldrich), 1% non-essential amino acids (NEAA) (Sigma-Aldrich), 1% Penicillin–Streptomycin (P/S) (Invitrogen, Carlsbad, CA, USA). 293FT cells (Invitrogen) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) with 10% FBS, 1% sodium pyruvate (Invitrogen), 1% NEAA, 1% Gln and 1% P/S.

An inducible human full length DISC1 SH-SY5Y cell line was generated by expression of non-mutant DISC1 in the tet-on retroTIGHT (Invitrogen) retroviral expression system. DISC1 expression was induced by adding doxycycline to the medium at a final concentration of 1 μg/ml.

Control and DISC1 knocked down SH-SY5Y stable cell lines were generated by expression of PLK0.1-Puro-CMV shRNA plasmids (Sigma-Aldrich) through lentiviral expression system (Sigma-Aldrich). The cells that expressed the constructs were selected after puromycin (Sigma-Aldrich) treatment (3 μg/ml)

Murine cortex and hippocampal primary neurons were prepared from 14 to 15 day embryos. Pregnant dams were euthanized by cervical dislocation in accordance with institutional guidelines for care and use of animals. The embryos were dissected in PBS Ca/Mg (Invitrogen) supplemented with 33 mM glucose. Pooled tissue was mechanically triturated, treated with trypsin (Invitrogen) and DNaseI (Roche Applied Science, Mannheim, Germany), and resuspended in Neurobasal medium (Invitrogen) supplemented with 50× B27 (Invitrogen), 0.55 g/100 ml glucose (Sigma-Aldrich), 42 mg/100 ml sodium bicarbonate (Sigma-Aldrich), 1% P/S and 1% glutamine. The cells were plated on poly-D-lysine-coated Petri dishes. Cultures were maintained in serum-free medium at 37°C in 95% air/5% CO_2.

DISC1 knockdown

DISC1 was silenced in SH-SY5Y cells using Mission® shRNA lentiviral transduction particles (Sigma-Aldrich, reference NM_018662) containing five alternative PLK0.1-Puro-CMV shRNA plasmids (Supplementary Material, Fig. S1 and Table S1). Mission® pLKO.1-puro non-mammalian shRNA particles (reference: SHC002V) were used as control. For DISC1 knockdown in primary neurons, lentiviruses were produced by calcium phosphate co-transfection of the shRNA constructs (DISC1 RNAi #1, Silenced N2, Silenced N3) (see Supplementary Material, Table S8**)** and the helper constructs VSVG and ΔR8.9 into 293FT packaging cells; virus-containing medium was collected 48 h after transfection, and added (10 ml of lentiviral solution/3 × 10^6 neurons) to the medium of primary neurons at 7 DIV. The medium was changed 24 h after infection, and incubation was continued for 72 h.

Protein extraction and western blot analysis

Cells were solubilized in lysis buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol

(DTT), 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 2 μM leupeptine, 400 μM phenylmethanesulfonylfluoride (PMSF), 50 μM β-glycerophosphate, 100 μg/ml trasylol). Protein content was determined by using the BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). Samples were subjected to SDS-PAGE and western blotting as described in Supplementary Methods. Antibodies used are described in Supplementary Material, Table S9. Protein band intensities were evaluated with Image J 10.2 software (National Institutes of Health, USA). All blots shown are representative of multiple independent experiments.

Proteomic studies

Proteomic analysis of cell lysates was performed by using high-resolution two-dimensional gel electrophoresis (2-DE) and Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) analysis of selected protein spots. A detailed description is available in the Supplementary Material.

Expression of VGF in brains from patients affected by schizophrenia, bipolar disorder, and major depression

Frozen BA6 cortical tissues from the Consortium Collection were obtained from the SMRI (Baltimore, MD) by C. K. (20). Tissue was homogenized in ice-cold 10% VRL buffer consisting of 50 mM HEPES pH 7.5, 250 mM sucrose, 100 mM KAc, 5 mM $MgCl_2$ and protease inhibitor (Roche Applied Science). Homogenates (2.5%) were subjected to SDS-PAGE, blotted on 0.2 μm nitrocellulose membrane (Whatman, Schleicher & Schuell, Germany), and probed with a VGF antibody sc-10383 (Santa Cruz Biotechnology Inc., CA, USA). Samples were randomized and run blind in several blots (15 samples in the same blot) and for normalization, blots were incubated with a GAPDH antibody. Densitometric analysis was performed with ImageJ 10.2 software. Band intensities of VGF signal were normalized to GAPDH signal.

Statistical analysis

Statistical analysis was performed with the IBM SPSS Statistic 20 package. For analysis of VGF in the SMRI samples, control brains were compared with brains of psychiatric patients by using a one-tailed unpaired Mann–Whitney *U*-test.

Genetic association of VGF with endophenotypes of the schizophrenia continuum

The genetic methods used here are described in detail in Supplementary methods. Briefly, we mined pre-existing genome-wide association data from studies of the Northern Finland Birth Cohort from 1966 (NFBC66) relating to scales derived from a psychometric questionnaire (23). To study the VGF gene region, 4 SNPs genotyped as part of the GWAS study were identified from a region 10 kb upstream to 10 kb downstream of VGF. Their SNP *P*-values for the three endophenotypes were extracted from the data from previous studies, including results from the analyses of (SAS) and (PHAS) conditioned on previously identified DISC1 risk-modulating variants. Association analysis had been performed by using PLINK (version 1.05) (24), and its additive linear regression model.

In order to distinguish between significant association and false discovery at the candidate gene level, multiple test correction was performed. The SNP spectral decomposition method was used, for multiple test correction, (25) with modifications by Li and Ji (26) to determine the effective number of independent marker loci (Meff) ($n = 3$). In addition to the number of markers, three variables were studied, and two of these measures were further analyzed in conjunction with DISC1. Although these measures and models are not completely independent of each other, we have included them in our multiple test correction in order to provide a conservative threshold for significance. Thus, the experiment-wide significance threshold required to keep type I error rate at 5% is $P = 0.05/15 = 0.0033$. To study how the pattern of associations related to each other and the VGF gene, haplotype blocks of LD were determined by using the HapMap CEU data according to the solid spine of LD criteria in the haploview program (27,28).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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