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ORIGINAL ARTICLE

Absence of evidence for bornavirus infection in schizophrenia, bipolar disorder and major depressive disorder

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In 1983, reports of antibodies in subjects with major depressive disorder (MDD) to an as-yet uncharacterized infectious agent associated with meningoencephalitis in horses and sheep led to molecular cloning of the genome of a novel, negative-stranded neurotropic virus, Borna disease virus (BDV). This advance has enabled the development of new diagnostic assays, including *in situ* hybridization, PCR and serology based on recombinant proteins. Since these assays were first implemented in 1990, more than 80 studies have reported an association between BDV and a wide range of human illnesses that include MDD, bipolar disorder (BD), schizophrenia (SZ), anxiety disorder, chronic fatigue syndrome, multiple sclerosis, amyotrophic lateral sclerosis, dementia and glioblastoma multiforme. However, to date there has been no blinded case–control study of the epidemiology of BDV infection. Here, in a United States-based, multi-center, yoked case–control study with standardized methods for clinical assessment and blinded serological and molecular analysis, we report the absence of association of psychiatric illness with antibodies to BDV or with BDV nucleic acids in serially collected serum and white blood cell samples from 396 subjects, a study population comprised of 198 matched pairs of patients and healthy controls (52 SZ/control pairs, 66 BD/control pairs and 80 MDD/control pairs). Our results argue strongly against a role for BDV in the pathogenesis of these psychiatric disorders.
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Keywords: Borna disease virus; infection; schizophrenia; affective disorders; pathogenesis

Introduction

The concept that infectious agents may cause mental illness has a long and complex history.[1–6] Although acute brain infections (for example, with herpes simplex or rabies virus) are clearly associated with sporadic cases of psychosis,[7–9] and others like influenza are historically associated with epidemic psychosis,[10–12] no linkage is established between infection and the majority of cases of affective disorders or schizophrenia (SZ). Infection-based models have often focused on specific microbes as potential culprits. One candidate is Borna disease virus (BDV), a noncytolytic RNA virus that causes movement and behavior disturbances in warm-blooded animal hosts from birds to primates.[13–15] BDV has been the subject of intensive research interest over the past 2 decades,[3,4] but findings are inconsistent. Recent reports of novel bornaviruses in birds (avian bornavirus (ABV))[16,17] and the discovery of BDV sequences in genomes of mammals, including humans,[18,19] have accentuated this interest.

Some studies note an increased prevalence of antibodies to BDV proteins in psychiatric patients, but rates vary substantially by patient groups (1.6–100%) and assay type. High seroprevalence rates are also found in controls (1.2–46%).[3,13] Serial analyses may yield higher seroprevalence rates,[20–22] though some

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studies fail to find differences between serially assessed patients and controls.[21] Some variability may be attributed to assay specificity. In chronic fatigue syndrome, 27 of 169 subjects with disease were seropositive by enzyme-linked immunosorbent assay (ELISA), but none (0/53) were positive by western immunoblot (WIB).[23] The highest reported rates in psychiatric patients were found using the methods designed to detect BDV-specific antigen–antibody complexes (up to 100% in affective disorder samples);[24] however, other investigators report these methods are nonspecific.[25] Results of the studies using molecular strategies are also inconclusive; however, differences in results are more difficult to ascribe to assay specificity given that research groups tend to use similar primer sequences and protocols.

Naturally infected horses, sheep, cattle, cats and birds could serve as reservoirs for the virus; however, there are no detailed epidemiological studies in animal populations and no studies demonstrating the transmission from domestic animals to humans. BDV is transmitted efficiently through contact with nerve terminals (for example, olfactory infection), but the observation that BDV may be present in peripheral blood cells suggests the potential for hematogenous infection. One study in Japan revealed that 4–5% of random blood donors had BDV nucleic acids in peripheral blood mononuclear cells.[26] The only study to report a higher prevalence of historical infection in healthy blood donors (30%) relied on a sandwich enzyme immunoassay to detect circulating immune complexes[24] that does not discriminate the presence of BDV antigen from nonspecific reactivity.[27] Limitations in our understanding of pathogenesis continue to restrict our ability to tailor a study design for the selection of best sampling compartment, timing of sample collection relative to illness onset or exacerbation and diagnostic markers for detection of infection.[3,28]

This multi-center, case–control investigation addressed these research gaps by subjecting prospectively collected samples from well-characterized patients with neuropsychiatric diseases and matched, healthy controls to blinded analysis using standardized methods for serological and molecular analysis. Our primary goal was to test for an association between infection and neuropsychiatric disorder, as determined by antibody and/or nucleic acid status. We also evaluated whether the infection is associated with specific clinical variables, using rigorous methods for diagnosis and assessment. The prevalence of serum antibodies to BDV and of BDV nucleic acids in peripheral white blood cells (WBC) was determined in patient-control pairs, matched on age, sex, geographical residence, socioeconomic status, and timing of blood draws.

Materials and methods

Human subjects

Patients with DSM-IV diagnoses[29] of SZ, bipolar disorder (BD) and major depressive disorder (MDD), ages 20–75 years, were recruited through outpatient clinics of University of California Los Angeles (UCLA) and University of California Irvine (UCI) using informed consent procedures, and protocols approved by the Institutional Review Boards of UCLA, UCI and Columbia University. We hypothesized that if a virus were associated with psychiatric disorder, evidence of infection would be detectable in peripheral blood during acute onset or exacerbation of pre-existing psychiatric illness, with peak virus-specific, IgG antibodies during convalescence, 6 weeks later. In keeping with this paradigm, for study inclusion, patients had to experience psychiatric illness onset or exacerbation within 6 weeks of study entry (T1) and be available for repeat evaluation and blood draw 6 weeks later (T2). Patients with unstable medical illness were excluded. Recruited patients nominated healthy social contacts, to serve as matched controls, of the same sex, same socioeconomic status, similar age (± 5 years) and residing in the same geographical region as the patient. Yoked control candidates who were blood relatives, or current or previous household or sexual partners of nominating case subjects, or who had unstable medical illness, were excluded. Controls with substance-use disorders were excluded, but substance-use disorders were not an exclusion for patients except for those with histories of intravenous drug abuse.

Subject characterization

Diagnoses of SZ, BD and MDD were established by Structured Clinical Interview for DSM-IV Axis I Disorders (SCID).[30] Illness severity ratings were derived using Clinical Global Impressions-Severity[31] and Global Assessment of Function scales.[29] The SCID was also used to establish the presence of substance-use disorders in patients and any exclusionary Axis I and Axis II diagnoses. Controls were screened using the Depression Rating Scale[32,33] for MDD and BD subjects; severity of mania was evaluated through the Young Mania Rating Scale.[34] SZ severity was rated using the Positive and Negative Symptoms Scale.[35] Semi-structured interviews provided standardized collection of exposure and demographic data; individual and family medical and psychiatric histories; medication history, including response to prior neuropharmacological trials[36–38] and current medications. At T2, severity ratings were repeated, and changes from T1 in physical status (intercurrent illness) or medications were recorded.

Sample collection and processing

Blood samples were first collected from patients within 6 weeks of onset of an acute episode or clinically significant exacerbation (T1), and 6 weeks later (T2) to allow for the determination of changes in viral nucleic acid load or antibody titers. T1 and T2 blood samples were collected from controls within 4 weeks of the respective T1 and T2 blood draws from the case to whom that control was yoked. Blood was collected into red-top tubes to obtain serum, and into blue-top tubes (3.2% sodium citrate) to obtain plasma

and WBC. All samples were coded to protect patient privacy and ensure blinded analysis. Samples were processed within 24 h of collection in a facility never used for BDV-related work (Supplementary Information).

Molecular assays

To minimize the potential for artifacts due to inadvertent introduction of BDV nucleic acid, PCR master mixes were prepared in different rooms than those used for PCR, and separate glove boxes and equipment were used for individual work steps (master mix, template addition, handling of positive/negative controls).

Quantitative real-time reverse transcriptase (RT)-PCR assays for BDV nucleoprotein (N) and phosphoprotein (P) gene sequences: real-time RT-PCR assays were established for the detection of strain V, He/80 and the newer isolate, No/98, that represent the most divergent BDV sequences. PCR primers for BDV N and P gene sequences of the designated strains, and for porphobilinogen deaminase (housekeeping gene), were designed using Primer Express 1.0 software (Applied Biosystems, Foster City, CA, USA). Template standards were cloned by PCR into vector pGEM-T easy (Promega, Madison, WI, USA) using viral RNA obtained from cultured cells infected with strain V, He/80 or No/98.

To allow the recognition of amplification products from synthetic template standards used in real-time RT-PCR assays, selective mutations were introduced to create specific restriction sites (see design of plasmids and restriction sites for use with He/80 and strain V primers and probes, and No/98 primers and probes, in Supplementary Information).

RNA was extracted from WBC using Tri-Reagent (MRC, Cincinnati, OH, USA). RNA quantity and quality were assessed by spectrometry and optical density 260/280 ratios. cDNA was synthesized from 2 μg of RNA from each sample using Taqman reverse transcription reagents (Applied Biosystems) (66 μl per well). RT reaction volume (5 μl, cDNA representing 150 ng of starting RNA) was combined with 2.5 μl of each primer and probe, and 12.5 μl of TaqMan Universal Master Mix (Applied Biosystems), and subjected to the following thermal cycling conditions (Model 7700 Sequence Detector, Applied Biosystems): 2 min, 50 °C; 10 min, 95 °C followed by 45 cycles of 1 min, 60 °C and 15 s, 95 °C. Each PCR plate included dilution rows of the respective cloned BDV plasmid standard, as well as the cloned housekeeping gene standard, and negative controls representing normal human leukocyte RNA (added at RT stage; BD Biosciences Clontech, San Diego, CA, USA) and human placenta DNA (added at PCR stage; Sigma, St Louis, MO, USA).

RNA from coded T1 WBC aliquots was tested in duplicate for the presence of P and N gene transcripts in randomized, blinded manner by two separate real-time RT-PCR analyses, using two different primer sets. Before beginning PCR assays, criteria for establishing the results as positive were defined as consistent detection of either P or N gene transcripts, using at least one of the two primer sets. In the event that discordant results were observed for the first two aliquots assessed by real-time RT-PCR, RNA extracted from a third WBC aliquot would be subjected to an additional round of real-time RT-PCR analysis. In this instance, samples would be designated positive or negative based on the two real-time RT-PCR results found to be concordant (for example, two of three negative real-time RT-PCRs would be designated a negative result; two of three positive real-time RT-PCRs would be designated a positive result).

Consensus PCR for ABV sequences. Following the identification of multiple novel bornaviruses in diseased psittacines,[16,17] we established new primer sets and assays for the detection of ABV genotypes 1–4 (Supplementary Information).

PCR products were size-fractionated on agarose gels, purified (Qiaquick PCR purification kit; QIAGEN, Valencia, CA, USA) and directly dideoxy-sequenced (Genewiz, South Plainfield, NJ, USA). Sequences were analyzed by GenBank BLASTn nucleotide search, in comparison with a 440-nt sequence for ABV 1–4 (GenBank accession numbers FJ169441, FJ169440, EU781967, GU249595), and alignment using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0.2.[39]

Serological assays

ELISA. Coded serum samples were assessed for the presence of antibodies to BDV proteins by ELISA as previously described.[40] Recombinant BDV proteins p40 (N) and p23 (P) were expressed from pET-BDV N and pET-BDV P plasmids (vector pET15b, Novagen, Gibbstown, NJ, USA) as His-tagged fusion proteins for use as antigens in ELISA serology experiments. Carbonic anhydrase (Sigma) served as negative control antigen. All proteins were confirmed as immunoreactive in ELISA with sera from experimentally infected rodents (rats) and primates (*Rhesus macaques*) before use in assays with human materials. Human T1 and T2 samples were tested in duplicate in 96-well microtiter plates (dilutions of 1:1000, 1:2000, 1:4000, 1:8000) using 80 ng per well of recombinant N, P or carbonic anhydrase (nonspecific protein target). Sera from experimentally infected *R. macaques* were used as positive control (1:8000 dilution) and normal human serum (Jackson Laboratories, West Grove, PA, USA) as negative control (1:1000 dilution). Negative control wells without primary antisera were included for calibration. Serum samples were considered reactive with a BDV protein if optical density reading at 450 nm was two standard deviations above the mean of their nonspecific reactivity with carbonic anhydrase. Sera positive by initial ELISA for either or both BDV proteins were subjected to a second round of ELISA. Sera positive in the first round of ELISA, but not reactive with the same proteins in repeat ELISA were considered negative. Samples were only considered

positive in ELISA if immunoreactive to both BDV N and P proteins; any sera found positive by ELISA would be subjected to WIB analysis. Reactivity with both BDV N and P proteins by WIB was required to confirm infection. Any sera positive by initial ELISA, but not reactive with the same proteins in WIB would be considered negative. If positive sera were found, these were serially diluted and retested in ELISA to establish antibody titers against individual BDV proteins (ELISA titer defined as endpoint dilution yielding optical density of 0.3).

Immunofluorescence assay (IFA). Baseline serum samples were analyzed by IFA in a multi-step strategy. Each test series was conducted in 24-well plates and included two human control serum samples defined in previously published work as positive and negative for anti-BDV antibodies.[41] Two independent raters evaluated the immunoreactivity of coded samples at each step. Samples were initially evaluated in a screening IFA (1:10 dilution) using C6 cells infected with BDV strain He/80. Subsequently, in a second round of screening, samples rated positive in the first step were tested (1:10 dilution) with both BDV-infected and uninfected C6 cells. Immunoreactivity of samples with infected C6 cells was compared with immunoreactivity with uninfected C6 cells to determine the specificity of binding to BDV proteins vs nonspecific nuclear staining; samples with punctate immunoreactivity observed only in infected C6 cells, and not in uninfected C6 control cells, were considered confirmed as screen-positive samples. All samples rated as positive in these two IFA screening steps were retested using infected and uninfected C6 cells to determine titer and avidity (immunoreactivity with 6 M urea vs no urea). To assess the consistency of results across multiple IFA assays, a set of 43 samples, comprising additional aliquots of samples characterized as positive as well as negative in the first two screening steps, was subjected to repeat IFA testing.

Statistical analyses

PASW Statistics 18.0.3 (IBM SPSS Statistics/Mac, Armonk, NY, USA) was used for all statistical analyses. Distributions were examined to ensure they did not deviate from normality; data meeting this criterion were used to derive paired-difference scores and were evaluated for each variable across diagnostic groups using one-way analysis of variance. Diagnosis-restricted patient/matched control group status served as the independent variable. For continuous data deviating from normal distributions, group comparisons and correlational analyses were conducted using Mann–Whitney U (nominal $\alpha = 0.05$). χ^2 analyses were performed for nominal data (two-tailed Fisher's Exact test for significance).

Results

Sample characteristics

The final study sample consisted of 198 matched case–control pairs (396 subjects) with data available at T1: 52 SZ and 52 matched controls (SZ-C); 66 BD, and 66 BD-C; and 80 MDD and 80 MDD-C (Table 1). A total of 151 pairs (302 subjects) also had T2 data available (48 SZ/SZ-C, 51 BD/BD-C and 52 MDD/MDD-C matched pairs). Mean age of subjects did not differ between or across diagnostic groups. The proportion of males was higher in SZ (82.7%) (hence their matched controls, 82.7%) than in the other patient groups (BD, 56.1%; MDD, 57.5%) or their respective matched control groups (BD-C, 56.1%; MDD-C, 57.5%; $P = 0.004$). Distributions of ethnicity, country of origin and measures of socioeconomic status (% below poverty level and mean household income based on residence zip code) were similar across groups (Table 1). Patients did not differ from respective matched controls in exposure to domestic pets; current use of caffeine, tobacco and recreational drugs; and foreign travel (Table 1). SZ subjects were less likely to consume alcohol than SZ-C subjects (21.2% vs 42.3%; $P = 0.037$).

Laboratory assays

Molecular assays. Real-time RT-PCR for BDV N and P gene sequences of multiple bornavirus strains: RNA from all T1 WBC samples were studied in multiple real-time RT-PCR assays designed for the detection of He/80, strain V and No/98 strains of BDV with sensitivity thresholds of <25 RNA copies (Supplementary Information). None of the samples showed evidence of N or P gene sequences by any of the real-time RT-PCR primer sets used (Table 2).

PCR for ABV sequences: Using a newly designed PCR assay for the detection of M and P gene sequences of ABV genotypes 1–4 with a sensitivity threshold of <100 RNA copies (Methods, Supplementary Information), we examined WBC-derived RNA samples from a subset of 97 patients (24 SZ, 33 BD, 40 MDD) with current (last 6 months) or historical exposure to birds and/or fowl (46 of 97, or 47.4%, or 51 of 97, or 52.5%, respectively). No sequences specific for ABVs were identified (Table 2).

Serologic assays. ELISA for the detection of antibodies to BDV proteins: All T1 and T2 serum samples were tested in duplicate for immunoreactivity to recombinant N, P and carbonic anhydrase (nonspecific protein target). No samples were positive for antibodies to BDV by ELISA at either sampling time point.

IFA: T1 serum samples were tested for binding to He/80-infected C6 cells. Eight sera samples were consistently immunoreactive (8 of 396, or 2%). These sera samples were tested for avidity by repeating the assay in the presence of 6 M urea. Four sera samples had high avidity (2 BD, 1 BD-C, 1 SZ-C). Four had low avidity (1 BD, 2 BD-C, 1 MDD).

Table 1 Subject characteristics

Subject characteristic	*Subject group*					
	SZ (n = *52*)	*SZ-C* (n = *52*)	*BD* (n = *66*)	*BD-C* (n = *66*)	*MDD* (n = *80*)	*MDD-C* (n = *80*)
Age						
Mean, in years (s.d.)	41.0 (10.0)	40.6 (11.3)	44.3 (11.4)	43.3 (11.8)	42.0 (13.2)	40.9 (13.1)
Minimum–maximum	23–61	20–65	22–75	23–71	21–75	20–74
Sex						
n (% male)	43 (82.7)	43 (82.7)	37 (56.1)	37 (56.1)	46 (57.5)	46 (57.5)
Ethnicity n *(% of each subcategory)*						
Other	0 (0.0)	0 (0.0)	2 (3.0)	0 (0.0)	0 (0.0)	0 (0.0)
Asian	3 (5.8)	2 (3.8)	3 (4.5)	4 (6.1)	4 (5.0)	5 (6.3)
Hispanic	10 (19.2)	11 (21.2)	4 (6.1)	4 (6.1)	6 (7.5)	8 (10.0)
African-American	8 (15.4)	7 (13.5)	15 (22.7)	14 (21.2)	13 (16.3)	11 (13.8)
Caucasian	31 (59.6)	32 (61.5)	42 (63.6)	44 (66.7)	56 (71.3)	56 (70.0)
Poverty level[a]						
Mean, % < poverty level	17.35	13.48	14.39	18.96	15.22	14.42
s.d.	7.45	9.77	8.91	13.77	10.04	8.71
Household income[a]						
Mean, in US dollars	43 445	53 010	52 553	47 547	49 692	50 370
s.e.m.	1890	2824	3516	3160	2518	2195
Domestic pet at home						
Any type, ⩾1 month (ever), *n*/total[b] (%)	47/52 (90.4)	48/52 (92.3)	60/65 (92.3)	57/63 (90.5)	73/77 (94.8)	64/76 (84.2)
Alcohol use						
n/total[b] (%)	11/52 (21.2)	22/52 (42.3)	15/65 (23.1)	18/62 (29.0)	18/77 (23.4)	20/76 (26.3)
Caffeine use						
n/total[b] (%)	48/52 (92.3)	44/52 (84.6)	51/65 (78.5)	45/62 (72.6)	50/77 (64.9)	52/76 (68.4)
Smoking						
Current smoker, *n*/total[b] (%)	39/52 (75.0)	11/52 (21.2)	29/65 (44.6)	10/62 (16.1)	18/77 (23.4)	9/76 (11.8)
Ex-smoker, *n*/total[b] (%)	4/52 (7.7)	13/52 (25.0)	12/65 (18.5)	16/62 (25.8)	21/77 (27.3)	20/76 (26.3)
Recreational drug use						
Prior 4 weeks, *n*/total[b] (%)	6/52 (11.5)	6/52 (11.5)	4/65 (6.2)	4/62 (6.5)	6/77 (7.8)	1/76 (1.3)
Foreign residence/travel						
>1 month (ever), *n*/total[b] (%)	19/52 (36.5)	27/52 (51.9)	37/65 (56.9)	30/62 (48.4)	45/77 (58.4)	48/76 (63.2)
Last 6 months, *n*/total[b] (%)	1/19 (5.3)	0/27 (0.0)	3/37 (8.1)	2/30 (6.7)	3/45 (6.7)	5/48 (10.4)

Abbreviatioins: BD, bipolar disorder; C, control; MDD, major depressive disorder; SZ, schizophrenia.
[a]% below poverty level and household income, by zip code of residence (United States Census Bureau, http://www.census.gov; Summary File 3).
[b]Total *n* defined as number of subjects reporting.

Table 2 Molecular assays, T1

Assay type	*Primer set detection*		*Subject group* n *with positive results (%)*					
	Bornavirus strain	*Gene sequence*	*SZ* (n = 52)	*SZ-C* (n = 52)	*BD* (n = 66)	*BD-C* (n = 66)	*MDD* (n = 80)	*MDD-C* (n = 80)
Real time RT-PCR	He/80	N gene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		P gene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Strain V	N gene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		P gene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	No/98	N gene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		P gene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Universal	N gene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		P gene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
PCR	Avian bornaviruses, genotypes 1–4	M gene	0 (0)[a]	ND	0 (0)	ND	0 (0)	ND
		P gene	0 (0)[a]	ND	0 (0)	ND	0 (0)	ND

Abbreviations: BD, bipolar disorder; C, control; MDD, major depressive disorder; N, nucleoprotein; ND, not determined; P, phosphoprotein; SZ, schizophrenia.
[a]SZ, $n = 24$; BD, $n = 33$; MDD, $n = 40$.

Discussion

Analysis of 198 patients with neuropsychiatric disease and their 198 pairwise-matched controls revealed no evidence of BDV association with psychiatric diagnosis at acute onset or exacerbation of illness, using a broad repertoire of molecular and serological assays. In addition, we found no molecular evidence of BDV infection in any subject. Serology varied by platform. ELISA was negative for all subjects at T1 and T2. IFA indicated the presence of high avidity antibodies in 4 subjects: 2 BD, 1 BD-C, and 1 SZ-C. However, follow-up WIB analysis found no immunoreactivity of any of these four samples to BDV N or P (data not shown).

Disparities between our results and prior studies may reflect differences in subject recruitment and sampling procedures. First, patients and controls were of similar age (±5 years), sex, ethnic/racial group, socioeconomic status, geographical residence and date of blood sampling, controlling for common factors potentially confounding both host risk of exposure, as well as the response of each individual to infection. Second, participants were rigorously diagnosed to validate the presence or absence of psychiatric illness (presence of the relevant DSM-IV diagnosis in SZ, BD and MDD groups, without other primary psychiatric disorders; absence of psychiatric and substance-use disorder in controls) and provide information about phenotypic differences that might be linked to altered viral risk (seasonality, melancholia, psychosis, treatment-resistance, course of illness). Potential exposures (for example, animals, substances, foreign travel) and risk factors for altered response to infection (personal or family medical history, including autoimmune disorders; medications) were also thoroughly characterized with standardized instruments. Third, the serial sampling incorporated into our study design—within 6 weeks of an acute flare of illness and again 6 weeks later—were uniquely informed by classical acute and chronic phase models of host response to viral infection. The study was thus poised to collect critical information that might shed light on the influences of cofactors on BDV measures, including time and clinical severity. It is also possible that North American subjects differ from other study populations in prevalence of exposure to bornaviruses for reasons as yet unknown.

An alternative explanation for differences in results obtained in this and other studies may be our methods for sample collection, processing and assay protocols designed to minimize the risk of sample contamination. Given the high degree of sequence conservation across BDV strains, and the resultant difficulties in distinguishing *bona fide* BDV sequences, all blood samples were processed in an independent laboratory using newly purchased tools and equipment devoted to this project, and stored in a dedicated freezer. The incorporation of more than one strategy for serological detection of antibodies to bornaviral proteins (ELISA, IFA), and inclusion of methods to measure the avidity

of detected antibodies (urea treatment in IFA), minimized the possibility that sensitivity of any particular assay was insufficient for the detection of low levels of anti-BDV antibodies. The establishment of assay-specific study definitions of positive and negative findings, before conducting any assays, mitigated against potential concerns regarding bias in assay interpretation.

All sera were negative by ELISA; the majority was negative by IFA. The four serum samples with positive signal by IFA were shown to be nonspecific in follow-up WIB studies. Immune complex analyses[24] performed in another laboratory showed no association between the presence of complexes and disease status (data not shown). Real-time RT-PCR of WBC-derived RNA, including samples from subjects with IFA signal, showed no evidence of BDV-related nucleic acids or ABV sequences. These findings are consistent with those of Wolff *et al.*,[25] wherein neither BDV antigen nor nucleic acids were detected in human blood samples previously reported as positive in circulating immune complexes enzyme-linked immunoassay. The greater consistency in the current study across multiple assay platforms and detection targets may relate to its large size, well-controlled study design, use of serial samples, efforts to minimize the risk of laboratory contamination and sample blinding strategy. We cannot exclude the possibility that BDV RNA or antibodies may be present in the cerebrospinal fluid of some patients with neuropsychiatric diseases. However, there are no replicated studies that show evidence of infection in brain or cerebrospinal fluid. The vast majority of reports of footprints of BDV infection in these diseases are based on the analyses of PBMC and sera. Recent reports identifying the incorporation of BDV sequences into the genome of humans and other mammals,[18,19] indicate that bornaviruses infected primates over 40 million years ago. Whether BDV has ever been pathogenic for humans, or altered function in clinically significant ways, remains unsolved. Nonetheless, using sensitive laboratory measures, this large, tightly controlled, case–control analysis provides powerful evidence that molecular and serological markers of BDV exposure are largely absent from the peripheral blood of all subjects, even during acute neuropsychiatric illness, and argues strongly against a role for BDV in the pathogenesis of SZ or mood disorders.

but also in the healthy controls

Conflict of interest

The authors declare no conflict of interest.

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References

1 Rott R, Herzog S, Fleischer B, Winokur A, Amsterdam J, Dyson W *et al.* Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders. *Science* 1985; **228**: 755–756.
2 Amsterdam J, Winokur A, Dyson W, Herzog S, Gonzalez F, Rott R *et al.* Borna Disease Virus: a possible etiologic factor in human affective disorders? *Arch Gen Psychiatry* 1985; **42**: 1093–1096.
3 Hornig M, Briese T, Lipkin WI. Borna disease virus. *J Neurovirol* 2003; **9**: 259–273.
4 Chalmers RM, Thomas DR, Salmon RL. Borna disease virus and the evidence for human pathogenicity: a systematic review. *QJM* 2005; **98**: 255–274.
5 Yolken RH, Torrey EF. Viruses, schizophrenia, and bipolar disorder. *Clin Microbiol Rev* 1995; **8**: 131–145.
6 Lipkin WI, Hornig M. Neurovirology. Microbes and the brain. *Lancet* 1998; **352**(Suppl 4): SIV21.
7 Schlitt M, Lakeman FD, Whitly RJ. Psychoses and herpes simplex encephalitis. *South Med J* 1985; **78**: 1347–1350.
8 Steinberg D, Hirsch SR, Marston SD, Reynolds K, Sutton NP. Influenza infection causing manic psychosis. *Br J Psychiatry* 1972; **120**: 531–535.
9 Goswami U, Shankar SK, Channabasavanna SM, Chattopadhyay A. Psychiatric presentations in rabies. A clinico-pathologic report from South India with a review of literature. *Trop Geogr Med* 1984; **36**: 77–81.
10 Menninger KA. Psychoses associated with influenza, I. General data: statistical analysis. *JAMA* 1919; **72**: 235–241.
11 Mednick SA, Machón RA, Huttunen MO, Bonett D. Adult schizophrenia following prenatal exposure to an influenza epidemic. *Arch Gen Psychiatry* 1988; **45**: 189–192.
12 O'Callaghan E, Sham P, Takei N, Glover G, Murray RM. Schizophrenia after prenatal exposure to 1957 A2 influenza epidemic. *Lancet* 1991; **337**: 1248–1250.
13 Lipkin WI, Schneemann A, Solbrig MV. Borna disease virus: implications for neuropsychiatric illness. *Trends Microbiol* 1995; **3**: 64–69.
14 Ludwig H, Bode L, Gosztonyi G. Borna disease: a persistent disease of the central nervous system. *Prog Med Virol* 1988; **35**: 107–151.
15 Stitz L, Krey H, Ludwig H. Borna disease in rhesus monkeys as a model for uveo-cerebral symptoms. *J Med Virol* 1981; **6**: 333–340.
16 Kistler AL, Gancz A, Clubb S, Skewes-Cox P, Fischer K, Sorber K *et al.* Recovery of divergent avian bornaviruses from cases of proventricular dilatation disease: identification of a candidate etiologic agent. *Virol J* 2008; **5**: 88.
17 Honkavuori KS, Shivaprasad HL, Williams BL, Quan PL, Hornig M, Street C *et al.* Novel borna virus in psittacine birds with proventricular dilatation disease. *Emerg Infect Dis* 2008; **14**: 1883–1886.
18 Horie M, Honda T, Suzuki Y, Kobayashi Y, Daito T, Oshida T *et al.* Endogenous non-retroviral RNA virus elements in mammalian genomes. *Nature* 2010; **463**: 84–87.
19 Belyi VA, Levine AJ, Skalka AM. Unexpected inheritance: multiple integrations of ancient bornavirus and ebolavirus/marburgvirus sequences in vertebrate genomes. *PLoS Pathog* 2010; **6**: e1001030.
20 Bode L, Ferszt R, Czech G. Borna disease virus infection and affective disorders in man. *Arch Virol Suppl* 1993; **7**: 159–167.
21 Rackova S, Janu L, Kabickova H. Borna disease virus (BDV) circulating immunocomplex positivity in addicted patients in the Czech Republic: a prospective cohort analysis. *BMC Psychiatry* 2010; **10**: 70.
22 Heinrich A, Adamaszek M. Anti-Borna disease virus antibody responses in psychiatric patients: longterm follow up. *Psychiatry Clin Neurosci* 2010; **64**: 255–261.
23 Evengård B, Briese T, Lindh G, Lee S, Lipkin WI. Absence of evidence of Borna disease virus infection in Swedish patients with Chronic Fatigue Syndrome. *J Neurovirol* 1999; **5**: 495–499.
24 Bode L, Reckwald P, Severus WE, Stoyloff R, Ferszt R, Dietrich DE *et al.* Borna disease virus-specific circulating immune complexes, antigenemia, and free antibodies—the key marker triplet determining infection and prevailing in severe mood disorders. *Mol Psychiatry* 2001; **6**: 481–491.
25 Wolff T, Heins G, Pauli G, Burger R, Kurth R. Failure to detect Borna disease virus antigen and RNA in human blood [letter]. *J Clin Virol* 2006; **36**: 309–311.

26 Kishi M, Nakaya T, Nakamura Y, Kakinuma M, Takahashi TA, Sekiguchi S *et al.* Prevalence of Borna disease virus RNA in peripheral blood mononuclear cells from blood donors. *Med Microbiol Immunol* 1995; **184**: 135–138.
27 Wolff T, Burger R, Kurth R. Re: Absence of Borna virus in human blood [reply to letter]. *J Clin Virol* 2006; **36**: 314.
28 Lipkin WI, Hornig M, Briese T. Borna disease virus and neuropsychiatric disease—a reappraisal. *Trends Microbiol* 2001; **9**: 295–298.
29 American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders*, 4th edn. Text Revision (DSM-IV-TR). American Psychiatric Press: Washington, DC, USA, 2000.
30 First MB, Gibbon M, Spitzer RL, Williams JBW. *Structured Clinical Interview for DSM-IV Axis I Disorders*. Biometrics Research: New York, NY, USA, 1996.
31 Guy W. *ECDEU Assessment Manual for Psychopharmacology*. U.S. Department of Health, Education and Welfare: Washington, DC, USA, 1976, pp 218–222.
32 Hamilton M. A rating scale for depression. *Neurol Neurosurg Psychiatry* 1960; **23**: 56–62.
33 Williams JBW. A structured interview guide for the Hamilton Depression Rating Scale. *Arch Gen Psychiatry* 1988; **45**: 742–747.
34 Young RC, Biggs JT, Zeigler VE, Meyer DA. A rating scale for mania: reliability, validity and sensitivity. *Br J Psychiatry* 1978; **133**: 429–433.
35 Kay SR, Opler LA, Lindenmayer JP. Reliability and validity of the positive and negative syndrome scale for schizophrenics. *Psychiatry Res* 1988; **23**: 99–110.
36 Amsterdam JD, Hornig-Rohan M. Treatment algorithms in treatment-resistant depression. In: Hornig-Rohan M, Amsterdam JD (eds). *Psychiatric Clinics of North America: Treatment-Resistant Depression*. W.B. Saunders: Philadelphia, 1996, pp 371–386.
37 Khot V, DeVane CL, Korpi ER, Venable D, Bigelow LB, Wyatt RJ *et al.* The assessment and clinical implications of haloperidol acute-dose, steady-state, and withdrawal pharmacokinetics. *J Clin Psychopharmacol* 1993; **13**: 120–127.
38 Van Putten T, Marder SR, Mintz J, Poland RE. Haloperidol plasma levels and clinical response: a therapeutic window relationship. *Am J Psychiatry* 1992; **149**: 500–505.
39 Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007; **24**: 1596–1599.
40 Briese T, Hatalski CG, Kliche S, Park YS, Lipkin WI. An enzyme-linked-immunosorbent assay for detecting antibodies to Borna disease virus-specific proteins. *J Clin Microbiol* 1995; **69**: 348–351.
41 Billich C, Sauder C, Frank R, Herzog S, Bechter K, Takahashi K *et al.* High-avidity human serum antibodies recognizing linear epitopes of Borna disease virus proteins. *Biol Psychiatry* 2002; **51**: 979–987.

Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)