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Detection of adeno-associated virus 2 and parvovirus B19 in the human dorsolateral prefrontal cortex

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Although animal parvoviruses have long been recognized as causes of brain pathology in multiple animal models, especially during early development, human parvoviruses are rarely thought of as neurotropic or causes of neuropathology in humans. However, several recent case reports have suggested possible associations of parvovirus B19 (B19) infection with various neurological and neuropsychiatric symptoms. Adeno-associated virus 2 (AAV2) is related to B19 but has thus far not been shown to be associated with any human disease but is of clinical interest because of the recent use of recombinant AAV vectors in human gene therapy, including gene delivery to the brain. To date, there have been no large-scale studies of the propensity of wild-type human parvoviruses to infect the brain. The Stanley Medical Research Institute Brain Collection offered a unique opportunity to study a large sample ($n = 104$) of dorsolateral prefrontal cortex (DLPC) DNAs isolated from unaffected control, schizophrenic, and bipolar disorder brains for the presence of parvoviral sequences. This is the first investigator-blinded study to document the presence of parvoviral sequences in the DLPC by utilizing highly sensitive nested polymerase chain reaction (nPCR) and DNA sequencing. Of the overall sample, 6.7% to 12.5% were positive for AAV2, and 14.4% to 42.3% were positive for B19 sequences, with no statistical differences among subgroups. This is the first report to demonstrate the presence of human parvoviruses in a large cohort of adult DLPC, which underscores the need to gain a better insight into the basic biology of parvovirus-brain interactions, including mechanisms of infection and persistence. *Journal of NeuroVirology* (2006) **12,** 190–199.

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Introduction

Parvoviruses are ubiquitous and among the smallest viruses known, measuring 18 to 26 nm in diameter. These viruses are naked and quite resistant to destruction by heat and chemical treatments. Parvoviruses contain a single-stranded DNA genome of approximately 5000 nucleotides (Muzyczka and Berns, 2001).

There are multiple members of the parvovirus family that infect many different types of organisms from insects (Bruemmer *et al*, 2005) and shrimp (Sukhumsirichart *et al*, 2005) to reptiles (Farkas *et al*, 2004) and mammals (Hueffer and Parrish, 2003). Animal studies lend substantial support for the role of parvoviruses in central nervous system (CNS) infection and pathology. Several studies have shown that infections with animal parvoviruses (Kilham and Margolis, 1975) lead to brain pathologies, including hemorrhagic encephalopathy, in rodents and cerebellar hypoplasia, with resulting cerebellar ataxia in various cat and rodent models. Newborn cats infected with the parvovirus feline panleukopenia

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virus develop cerebellar ataxia (Johnson *et al*, 1967) due to cell death of granular and Purkinje cells, leading to cerebellar hypoplasia.

The mouse parvovirus, minute virus of mice strain i (MVMi), was found to infect cerebellar cells of newborn mice that were inoculated intranasally (Ramirez *et al*, 1996). The majority (70%) of these mice displayed typical cerebellar signs of intention tremor, ataxic gait, and sluggishness. A minority (8%) also displayed dwarfism. Early in the infection, virus was found to replicate in the laterodorsal thalamic and pontine nuclei. At the peak of infection, when the animals were clinically symptomatic, virus was found replicating in the dentate gyrus of the hippocampus, blood vessel endothelial cells throughout the parenchyma, the subventricular zone of the lateral ventricle, and the internal granular layer of the cerebellum. Histopathological analyses of the brains of MVMi-infected mice consistently showed a hypoplastic internal granular layer of the cerebellum, a slightly narrower molecular layer, and an uneven cellular alignment and less apparent cell bodies in the Purkinje cell layer.

Infection with rat virus (RV), a murine parvovirus, that also induces cerebellar signs has been shown to produce "mongolism" in hamsters when pups were either infected as newborns or with a sublethal dose of virus on neonatal day 4 but not at a later time in development (Kilham, 1961). These animals had broadened facial features, were smaller than uninfected littermates, and demonstrated a lowered learning ability (Landauer *et al*, 1967). Multiple studies with animal parvoviruses have shown that brain disorders can occur with some delay after birth as the result of intrauterine infection (Kilham and Margolis, 1975), reminiscent of the delays seen with the pervasive developmental disorders (e.g., autism) and possibly schizophrenia and bipolar disorder (American Psychiatric Association, 2000), and that the timing of infection during development is critical in determining the ultimate physical and behavioral outcomes.

Two members of the parvovirus family of human interest are parvovirus B19 (B19) and adeno-associated virus (AAV2). B19 is a clinically significant pathogen whose target is the erythroid progenitor cell in the bone marrow. B19 causes known human disease varying from the common childhood rash, erythema infectiosum or fifth disease, to potentially life-threatening conditions in the immunocompromised and those with underlying erythroid disorders. B19 causes transient aplastic crisis in individuals with disorders such as sickle cell anemia and hereditary sphrerocytosis as well as pure red cell aplasia in the persistently infected immunocompromised (e.g., transplant recipients and HIV+ individuals; Young and Brown, 2004; Heegaard and Brown, 2002). Infection with B19 has also been determined to cause arthritis, especially in middle-aged females (Kerr, 2000; Woolf *et al*, 1989). AAV2 infects humans but has hitherto not been associated with any human disease. AAV2, because of its nonpathogenic nature and its ability to integrate into the human chromosome in a site-specific manner (Kotin *et al*, 1992), has gained prominence as a recombinant vector for human gene therapy, including the central nervous system (Burger *et al*, 2005).

Recently, several case reports and series have been published that document a possible association of B19 infection with neurological and neuropsychiatric symptoms and sequelae. The bulk of the evidence links B19 with meningoencephalitis (Nolan *et al*, 2003; Kerr *et al*, 2002; Barah *et al*, 2001; Skaff and Labiner, 2001; Wierenga *et al*, 2001; Yoto *et al*, 2001; Druschky *et al*, 2000; Heegaard *et al*, 1995; Balfour *et al*, 1970). Of the 12 cases documented by Kerr *et al* (2002), 5 were reported to have long-term psychiatric sequelae: 1 displayed mental retardation and developmental delay; 1 showed cognitive deficits and personality change (from previously placid to unpredictably aggressive and violent); 1 had an altered affect; 1 demonstrated cognitive deficits, bouts of agitation, mood instability, and mixed anxiety symptoms (phobias and panic attacks); and the 5th case showed developmental delay (whereas the twin had cerebral palsy and seizures). Other neurological disorders with a possible association with B19 infection are stroke (Mandrioli *et al*, 2004; Wierenga *et al*, 2001), meningitis (Tabak *et al*, 1999; Koduri and Naides, 1995; Okumura and Ichikawa, 1993), encephalopathy (Umene and Nunoue, 1995), vasculitic encephalopathy (Bilge *et al*, 2005; Bakhshi *et al*, 2002), seizures (Hsu *et al*, 2004; Skaff and Labiner, 2001), cerebellar ataxia (Shimizu *et al*, 1999), transverse myelitis, neuropathy, brachial plexitis, Guillain-Barré, and carpal tunnel syndrome (Barah *et al*, 2003; Torok, 2001).

B19 infection of the fetus can lead to a severe anemia called hydrops fetalis (Young and Brown, 2004). B19 DNA has been detected in multinucleated giant cells and endothelial cells of the brain of one hydropic fetus (Isumi *et al*, 1999), thus showing some potential to infect the human brain. Because B19 is a TORCH agent (i.e., capable of infecting the fetus), a recent study (Buka *et al*, 2001) evaluated B19 as a possible cause of psychosis. This study of the Providence, RI, cohort of the Collaborative Perinatal Project did not find a significant difference in the levels of immunoglobulin G (IgG) antibodies to B19 in mothers whose offspring went on to develop psychosis compared to matched controls; however, it was noted that if the sample size ($N = 27$) had been larger, the values may have reached significance. Another limitation was the heterogeneity of the population in terms of diagnoses (schizophrenia, affective psychosis, and other psychotic illnesses). Also, IgG levels measured at one time point at the end of pregnancy may not reflect infection of the fetus. Thus, further studies are warranted to clarify the possible role of B19 in the pathogenesis of disorders such as schizophrenia.

B19 infections occur commonly in the winter and spring months (Torok, 2001). Schizophrenia births have been shown to increase approximately 10% during the late winter and early spring months (Mortensen *et al*, 1999). A viral etiology of schizophrenia and bipolar disorder has been hypothesized (Yolken and Torrey, 1995). The dorsolateral prefrontal cortex (DLPC) is one of several brain regions implicated in the pathogenesis of schizophrenia and bipolar disorder (Selemon and Rajkowska, 2003). Although one study documented 1 of 12 human brain samples as being positive for AAV (Gao *et al*, 2004), there have been no large-scale, blinded studies to determine whether wild-type parvoviruses infect the brain. In this study, brain DNAs collected from the DLPC of schizophrenic, bipolar, and unaffected control subjects were tested for the presence of B19 and AAV2 sequences using nested PCR to investigate the potential for parvoviruses to infect the brain and potentially be associated with chronic mental illnesses such as schizophrenia and bipolar disorder.

Results

AAV2 and B19 nested primers are specific for their respective template targets

Because PCR, particularly nested PCR (nPCR), is very sensitive, it was first necessary to ensure that the nested primers were specific for AAV2 and B19, and that there was no cross-amplification. As demonstrated in Figure 1, only AAV2- or B19-specific amplification occurred when AAV2 (Figure 1A) and B19 NS1 (Figure 1B) primer sets were utilized, and no cross-amplification occurred. Both primer sets amplify regions found within the nonstructural replication genes, Rep for AAV2 and NS1 for B19. Similar results were seen for the B19 VP1 (capsid) primer set (data not shown).

Evidence of human specificity, lack of contamination, and intactness of DNA templates

Figure 2A, B, and C show that AAV2 and B19 sequences could not be amplified from mouse genomic DNA supporting human specificity, and a general laboratory contamination with parvoviral sequences could be readily ruled out. It should be noted that multiple, low-intensity bands were often visible even in the range of the expected size, but only high-intensity bands were considered positive and found to be consistent. It was also necessary to ensure that those array collection samples that were negative for PCR amplification of parvoviral sequences contained intact DNA. Array collection samples that were negative by nPCR for AAV2 and B19 sequences were tested for amplification of the human β-globin sequence that was readily amplified (data not shown).

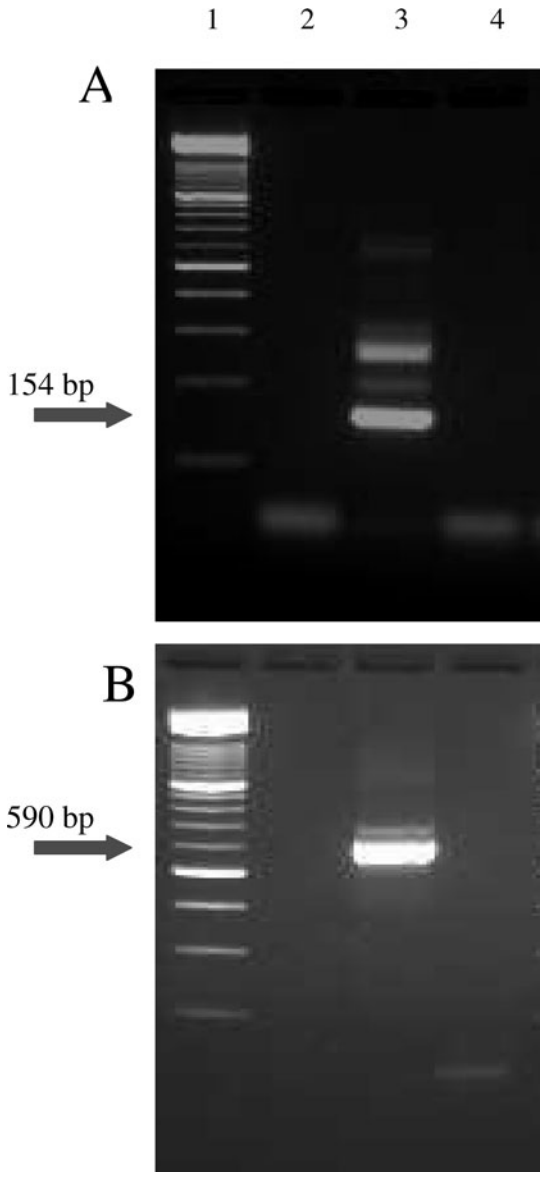

Figure 1 AAV2 nested primers are specific for AAV2, and B19 NS1 nested primers are specific for B19. **A**, AAV2 (Rep) nested primers amplify a 154-bp fragment; EtBr-stained gel of the nPCR products. Lane 1: 100-bp molecular weight (MW) marker; lane 2: negative control (H_2O instead of template); lane 3: pKY-10A (5 pg), containing the AAV2 full-length sequence, as template; lane 4: pYT103c (5 pg), containing the B19 full-length sequence, as template. **B**, B19 NS1 nested primers amplify a 590-bp fragment; EtBr-stained gel. Lane 1: 100-bp MW marker; lane 2: negative control (H_2O); lane 3: pYT103c (5 pg); lane 4: pKY-10A (5 pg). Bands less than 100-bp were considered primer-dimers. Bands greater than expected size were considered nonspecific amplification or carry-over from the outer PCR reaction.

AAV2 and B19-specific sequences can be amplified from the dorsolateral prefrontal cortex of human brain

Because it was not known with certainty whether parvoviruses can infect and persist in the human brain, it was important to examine whether AAV2 and B19 sequences could be found in human brain. Initially,

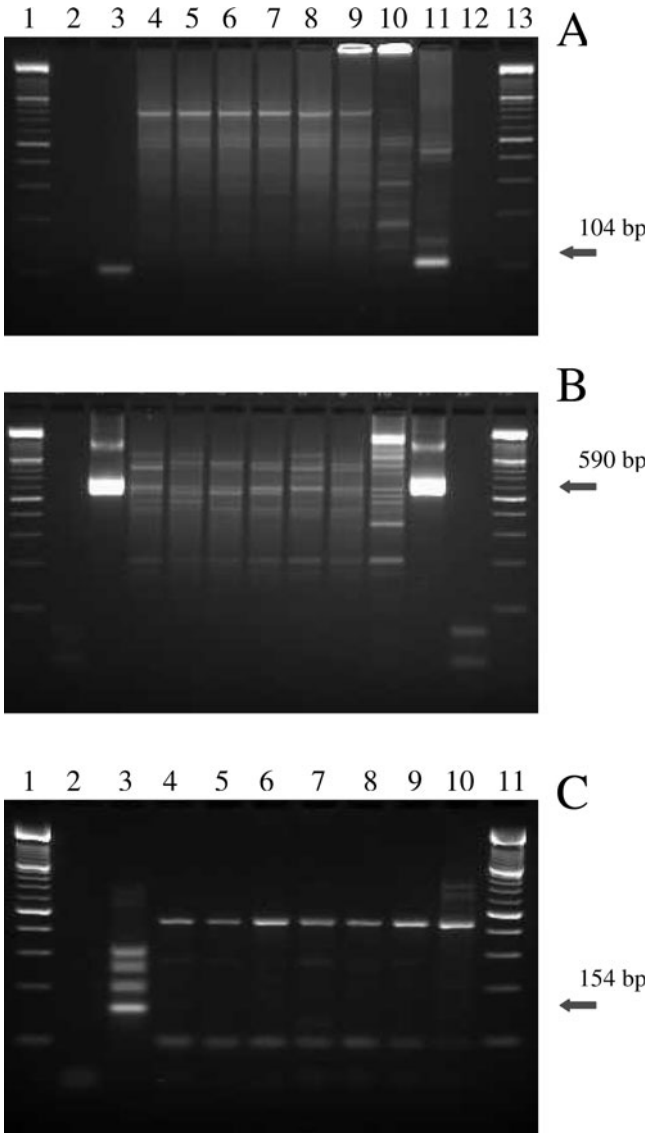

Figure 2 Lack of amplification of AAV2 or B19 sequences by nPCR from mouse tail genomic DNA. **A**, B19 VP1 nPCR of mouse tail samples; EtBr-stained gel. Lanes 1 and 13: 100-bp MW marker; lanes 2 and 12: negative control (H_2O); lanes 3 and 11: pYT103c; lanes 4–10: mouse tail genomic DNA from seven different mice. **B**, B19 NS1 nPCR of mouse tail samples; EtBr-stained gel. Lanes 1 and 13: 100-bp MW marker; lanes 2 and 12: negative control (H_2O), lanes 3 and 11: pYT103c; lanes 4–10: mouse tail genomic DNA from seven different mice. **C**, AAV2 nPCR of mouse tail samples; EtBr-stained gel. Lanes 1 and 11: 100-bp MW marker; lane 2: negative control (H_2O); lane 3: pKY-10A; lanes 4–10: mouse tail genomic DNA from seven different mice.

Southern blot analyses of the DLPC DNAs (10 μg per sample) were undertaken on approximately half of the samples ($n = 60$; half of the samples were assessed due to the blinded nature of the study), but this technique was not sensitive enough to detect these sequences (data not shown). However, as can be seen in Figure 3, AAV2 (Figure 3A) and B19 (both NS1 in Figure 3B and VP1 in Figure 3C) sequences could be amplified from human DLPC (BA46). Some representative nPCR products were transferred to membranes and probed with full-length viral probes for confirmation (data not shown).

The overall results of the screening of the Stanley Array Collection ($n = 104$) are shown in Table 1 and Figure 4. Those results of samples positive for both viral sequences (double positives) are shown in Table 2. To summarize, 6.7% to 12.5% were AAV2 positive, 14.4% to 42.3% were B19 positive, and 0.96% to 4.8% were double positive in the overall group ($n = 104$). AAV2 amplification was found in 14.3% to 20% of unaffected controls, 2.9% in schizophrenics, and 2.9% to 14.7% in bipolars. The difference among the groups missed statistical significance ($P = .056$) for AAV2 positivity. B19 amplification was found in 14.3% to 51.4% of unaffected controls, 8.6% to 34.3% in schizophrenics, and 20.6% to 41.2% in bipolars. The difference among groups was not statistically significant ($P = .353$). Of the unaffected controls 2.9% to 11.4% were double positive for AAV2 and B19. Of the bipolar subjects 2.9% were double positive. None of the schizophrenic subjects was found to be double positive.

Several of the PCR products ($n = 2$ for AAV2 with one sample performed in duplicate; $n = 6$ for B19 VP1; $n = 4$ for B19 NS1) were sequenced and compared to known sequences using BLAST (see Materials and Methods). The positive control nPCR product for AAV2 was 99% identical (data not shown) to a known AAV2 sequence (Srivastava *et al*, 1983). One AAV2 nPCR product from an unaffected control was also 99% identical to the known sequence (amplification, extraction, and sequencing performed in duplicate; data not shown). The other AAV2 nPCR product from a schizophrenic subject that was sequenced showed 98% identity with a C to T change at nucleotides (nt) 1541 and 1607. These changes were seen from sequencing off the forward and reverse primers (data not shown).

The positive control nPCR products for B19 VP1 and NS1 were 100% identical to known B19 sequences (Shade *et al*, 1986 for VP1; Zhi *et al*, 2004 for NS1; data not shown). Of the six B19 VP1 nPCR products, five showed sequence identity of 97% and the sixth showed 98% identity when compared to the same known sequence. Interestingly, all six showed a T to C change at nt 3223, and five of six showed a G to C change at nt 3219 (data not shown). These changes were seen on sequencing from the forward and reverse primers in each case so are likely not due to PCR-mediated mistakes. One-half of the products (three of six) were amplified, extracted, and sent for sequencing on one date, and the other samples were amplified, extracted, and sent for sequencing 2 weeks later, and given that the brain nPCR product sequences differ from the positive control, it is unlikely that the results are due to a plasmid contaminant. From the limited number of samples sequenced, there did not appear to

Table 1 Overall results of screening 104 Stanley Brain Array Collection DNA samples[a]

	AAV2+	*AAV2 equivocal*	*Range AAV+*	*B19+*	*B19 equivocal*	*Range B19+*
% positive, all subjects ($n = 104$)	7/104 (6.7%)	6/104 (5.8%)	6.7–12.5%	15/104 (14.4%)	29/104 (27.9%)	14.4–42.3%
Unaffected controls ($n = 35$)	5/35 (14.3%)	2/35 (5.7%)	14.3–20%	5/35 (14.3%)	13/35 (37.1%)	14.3–51.4%
Schizophrenia ($n = 35$)	1/35 (2.9%)	0/35 (0%)	2.9%	3/35 (8.6%)	9/35 (25.7%)	8.6–34.3%
Bipolar disorder ($n = 34$)	1/34 (2.9%)	4/34 (11.8%)	2.9–14.7%	7/34 (20.6%)	7/34 (20.6%)	20.6–41.2%

Q1 [a] Range: lower value is AAV2+ or B19+; higher value is the sum of + and equivocals (see Materials and Methods). AAV2+ and B19+ columns correspond with the first and second bars in Figure 4, respectively, for each subgroup.

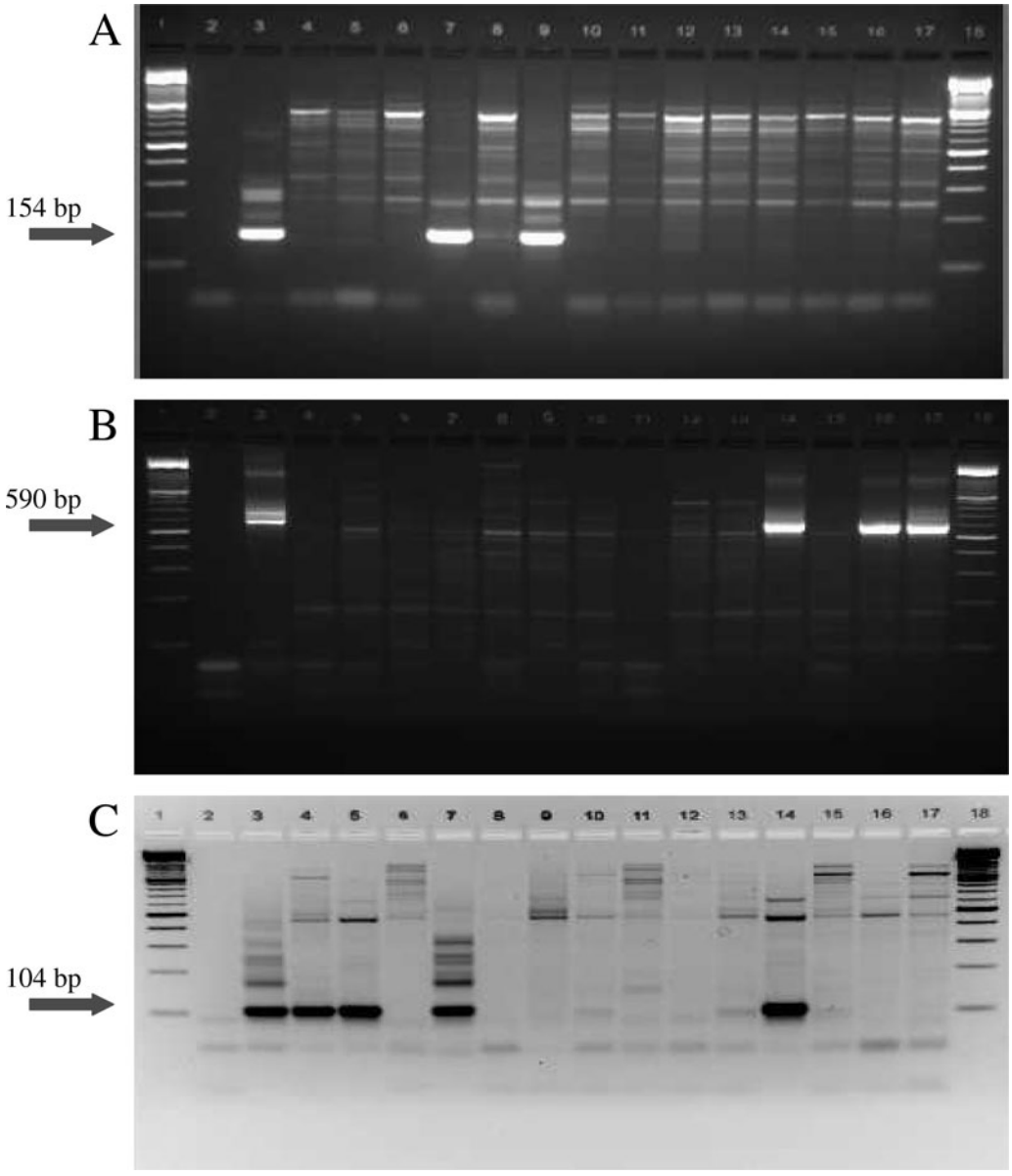

Figure 3 Representative (**A**) AAV2, (**B**) B19-NS1, and (**C**) B19-VP1 nested PCR screening of Stanley Brain Array Collection DNA samples. In each figure, lanes 1 and 18 are the 100-bp MW marker; lane 2 is the negative control (H_2O); and lanes 3 to 17 are representative array collection samples. Samples are not matched from one gel to the next.

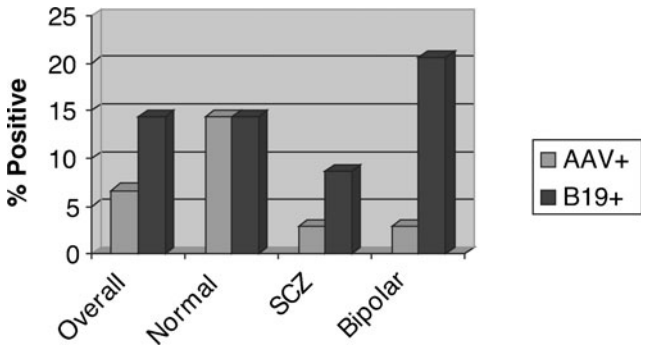

Figure 4 Graphical representation of the AAV+ and B19+ columns in Table 1. SCZ = schizophrenia.

be any disease-specific association with the sequence changes.

Of the four B19 NS1 nPCR products, one showed 100% identity (data not shown) to the positive control. The other three showed 99% identity with all containing consistent changes at nts 1710 (T to C), 1884 (G to A), and 1929 (G to T). One of the three also showed a change at nt 1815 (data not shown). Again, from the limited number of samples sequenced, there did not appear to be any disease-specific association with the sequence changes.

The overall study population consisted of approximately 2:1 males to females. AAV2+ males outnumbered females by a ratio of 6:1, but this was not statistically significant ($P = .448$). Also, B19+ females outnumbered males by a ratio of 1.5:1, but did not reach statistical significance ($P = .052$).

Discussion

This study of 104 human DLPC DNAs was undertaken to determine whether human parvoviral, specifically AAV2 and B19, DNA sequences could be amplified from the human brain and determine if any associations of these viruses with schizophrenia and bipolar disorder could be documented. The results clearly demonstrate that both AAV2 and B19 sequences can be specifically amplified from the human DLPC by nPCR (products confirmed by sequencing). Thus, given the significant size of the test sample and with investigator blinding, the results provide substantial support for parvoviral infection of the human brain.

One limitation of this study was that equivalent amounts of DNA were utilized in each PCR reaction, not DNA from equivalent numbers of cells. Given that schizophrenic and bipolar brain tissues may have contained fewer cells (Jarskog *et al*, 2005; Manaye *et al*, 2005; Thompson *et al*, 2001), the chance of amplifying parvoviral sequences could have been lower in these groups due to reasons unrelated to parvovirus pathology. The power of the study was well below the desired power of 0.8. A larger sample size would have been helpful to determine if there is indeed a difference in parvovirus infectivity in these mentally ill groups; however, the opportunity to analyze such a large, well-characterized sample of brain DNA has provided a great insight into parvovirus biology in the central nervous system in general.

A viral etiology of schizophrenia and bipolar disorder has been hypothesized for over a century (Yolken and Torrey, 1995). Proving such a hypothesis for diseases that likely have their origin in fetal or early childhood yet do not overtly present until decades later is a daunting task. Most studies to date have had to rely on looking for candidate viruses or an immune response to viruses in the body fluids and tissues (e.g., blood and cerebrospinal fluid [CSF]) of adults diagnosed as having these disorders. The obvious problem is that the virus and/or the immune response may not be present after many years.

As with most human diseases, schizophrenia and bipolar disorder will likely prove to be multifactorial in nature. For instance, it may be that these disorders only manifest in those individuals and families that have an underlying susceptibility to a causal infectious agent. Although this study does not provide evidence linking parvoviruses to schizophrenia or bipolar disorder, the data do suggest that these viruses infect and persist in the brain, and thus have the opportunity to interact with the different genetic backgrounds of these unique populations. Schizophrenia and bipolar disorder are not homogeneous disorders.

Table 2 Double-positive results[a]

	Double+	*Double equivocal*	*AAV2+/B19 equivocal*	*B19+/AAV2 equivocal*	*Range*
% positive, all subjects ($n = 104$)	1/104 (0.96%)	2/104 (1.9%)	1/104 (0.96%)	1/104 (0.96%)	0.96–4.8%
Unaffected controls ($n = 35$)	1/35 (2.9%)	2/35 (5.7%)	1/35 (2.9%)	0/35 (0%)	2.9–11.4%
Schizophrenia ($n = 35$)	0/35 (0%)	0/35 (0%)	0/35 (0%)	0/35 (0%)	0%
Bipolar disorder ($n = 34$)	0/34 (0%)	0/34 (0%)	0/34 (0%)	1/34 (2.9%)	2.9%

[a]Double+ = positive for both AAV2 and B19. Equivocal samples (see Materials and Methods). Range: lower value is double+; higher value is the sum of all values in the row.

Each disorder has already been subtyped (American Psychiatric Association, 2000), and will likely be further subdivided into categories, each with its own etiologies. As these disorders may have their origin in fetal life, studies of adult brain may not provide the evidence needed to link viruses in general and parvoviruses in particular to chronic mental illness. Further studies are needed to determine the exact nature of the interactions of parvoviruses with the central nervous system, especially the developing brain, and any long-term sequelae.

Interestingly, there was little overlap in AAV2 and B19 positivities in individual subjects (i.e., there were very few double positives even when equivocal subjects were included). The significance in terms of virus-host and virus-virus interactions is unknown. Though not impossible, the results would suggest that both viruses rarely persist in the same individual brain. There is the question of whether AAV can promote the lytic life cycle of B19 and vice versa. There is also the question of whether AAV alters the B19 receptor status and vice versa.

Wild-type AAV is known to integrate *in vitro* into the human chromosome in a site-specific fashion (Kotin *et al*, 1992), thus forming a latent infection. The ability of wild-type AAV to integrate into the human chromosome *in vivo* has not been shown to date, but AAV can persist in the episomal form (Schnepp *et al.*, 2005; Song *et al*, 2001). There have been some reports of B19 persisting in tissues such as synovial (Hokynar *et al*, 2000), skin (Nikkari *et al*, 1996), myocardial (Kuhl *et al*, 2005), and bone marrow (Cassinotti *et al*, 1997), but by an as yet unknown mechanism. In prior studies, B19 was found in the brain of one hydropic fetus following acute infection of the mother (Isumi *et al*, 1999), and AAV was found in 10% (of 12 tested samples) of human brain (Gao *et al*, 2004), but it was unknown whether these viruses would be present in the adult brain of subjects not known to have been acutely exposed to B19 or AAV. Most persons, based on serological studies, have been exposed to AAV (60% to 70% by age 19; Erles *et al*, 1999) and B19 (50% by age 15; Young and Brown, 2004) by early adulthood with the majority being exposed in childhood. Therefore, it is unlikely that the study sample was acutely infected by AAV and/or B19 (average age for all subjects was in the mid-40s), and the results of this study suggest persistent infection in the brain by both AAV2 and B19. This study was limited by a lack of matched serum to determine anti-AAV2 and anti-B19 immune status that would have provided a determination of acute versus past infection. An important question, however, is whether those persons that have persistent parvovirus infection of the brain are able to mount adequate immune responses to these viruses.

The main limitation of this study was the use of nPCR to determine the presence of parvoviral sequences. The possibilities of contamination and false positives/negatives are a concern. Even though every effort was made to optimize the reactions and control contamination in the laboratory (see Materials and Methods), the investigator could not control possible sources of contamination as the brain tissues were preserved and DNA isolated at another laboratory unknown to the investigator. However, as Southern blot analysis was not sensitive enough to detect these viral sequences (data not shown), it is likely that a small, perhaps select group of cells become infected with and allow persistence of the parvoviruses; therefore, nPCR was the preferred method as has been used previously in the search for other viruses that may be associated with schizophrenia (Taller *et al*, 1996). Given that the sequences obtained for the nPCR products were often different from the plasmid control, the likelihood of plasmid contamination of brain DNA samples is diminished. In fact, the results with the small number of products sequenced suggest that brain-specific parvoviral sequences may exist. Whether distinct human brain-specific serotypes, as well as disease-specific serotypes, exist should be further explored, even though the limited data presented here does not support disease-specific serotypes. Larger-scale sequencing studies are warranted on more samples.

The natural route of infection and the mechanism by which wild-type parvoviruses infect the brain are not known. Whether the brain receptors for parvoviruses are the same as those found on other target cells is also not known. For instance, B19 is believed to enter erythroid-type cells via interactions with P antigen (globoside; Brown *et al*, 1993) and $\alpha 5\beta 1$ integrin (Weigel-Kelley *et al*, 2003). More recently, another coreceptor has been identified on erythroid-type cells called Ku80 (Munakata *et al*, 2005), and the role of Pantigen has been questioned (Kaufmann *et al*, 2005). AAV2 has been shown to utilize heparan sulfate proteoglycan (HSPG; Summerford and Samulski, 1998) and the fibroblast growth factor receptor 1 (FGFR1; Qing *et al*, 1999) as well as $\alpha V\beta 5$ integrin (Summerford *et al*, 1999) as coreceptors for entry. At least one study supports the role of FGFR1 in AAV entry *in vivo* in the rat brain (Hadaczek *et al*, 2004). Further studies are warranted to determine what role, if any, these molecules play in the life cycle of wild-type AAV2 and B19 in the human brain.

Understanding parvoviral persistence is also of great interest. A better, broader understanding of the interaction between parvoviruses and the various tissues they can infect is needed. In fact, the full extent of which tissues can be infected naturally by parvoviruses, especially B19, is not known. Gaining improved knowledge of the extent of parvoviral-host interactions once persistence is established is a current focus of investigation.

As has been noted previously (Gao *et al*, 2005), the presence of wild-type parvoviruses *in vivo*, particularly AAV, has implications for gene therapy. The possibility of recombination between vectors and wild-type virus is a serious concern. The long-term effects

on both gene delivery and host cell homeostasis are not known. Further studies are warranted to better understand the interaction between persistent parvoviruses and gene therapy vectors.

As noted in the introduction, the animal parvoviruses often cause cerebellar pathology and dysfunction when infections occur during early development. Parvovirus B19 has been associated in one case report with cerebellar ataxia (Shimizu *et al*, 1999). Cerebellar involvement was also noted in four (clinically in two; at autopsy in two) cases of B19-associated meningoencephalitis in infants and children (Kerr *et al*, 2002). Torok has also discussed possible cerebellar defects following *in utero* B19 infection (Torok, 2001). The current studies in DLPC may only provide a narrow view of parvovirus-brain interactions. Investigations are ongoing to determine what role parvoviruses, particularly B19, may play in cerebellar pathology and dysfunction in humans, especially during early development.

Materials and methods

Stanley Brain Array Collection DNA samples
Twenty-microgram aliquots of DNA isolated from the DLPC (BA46) of 105 subjects (Torrey *et al*, 2000) were provided by the Stanley Medical Research Institute (SMRI). The DNA was isolated using a Promega Wizard genomic DNA extraction kit. Of the 105 subjects, 35 were unaffected controls, 35 were schizophrenic, and 34 suffered from bipolar disorder; one case was omitted by the SMRI. All samples were provided in a blinded fashion to the investigator. The blind was not broken until the nPCR data were submitted to SMRI. The samples were matched for age; the average age was 44.2, 42.6, and 45.4 for unaffected controls, schizophrenics, and bipolars, respectively. The sex ratio was the same for the unaffected and schizophrenic groups (26M:9F). The bipolar group contained 16 males and 18 females (16M:18F). All subjects were white except for one Black and one Native American in the bipolar group and one Hispanic in the schizophrenic group.

Cell lines and mice
B19p6-EGFP transgenic mice were produced on the C3H background. These mice express enhanced green fluorescent protein (EGFP) driven by the B19 promoter at map unit 6 (p6). All procedures with mice were approved by the University of Florida College of Medicine IACUC.

DNA isolation
Genomic DNA was isolated from mouse tail snips as previously described (Sambrook and Russell, 2001).

Polymerase chain reaction (PCR)
nPCR amplification of AAV2 and B19 NS1 and VP1 sequences was performed using previously described primers (Tobiasch *et al*, 1998, for AAV2; Schennach *et al*, 2002, for NS1 and Barah *et al*, 2001, for VP1) and puReTaq Ready-To-Go PCR Beads (GE Healthcare) per the manufacturer's instructions. Approximately 1 μg of genomic DNA (equivalent amounts for each brain collection sample) and 5 pg of plasmid DNA were utilized as templates. Plasmids included pYT103c (Shade *et al*, 1986) that contains the full-length B19 genome with hairpin deletions and pKY-10A that contains the full-length AAV2 genome except for the inverted terminal repeats as positive controls. Thermocycler parameters were 95°C for an initial 2 min. denaturation followed by 35 cycles: 95°C for 1 min, 55°C for 1 min, 72°C for 1 min; followed by a final 72° extension for 4 min. The AAV2-specific primers produced a product of 154 bp; the B19 NS1-specific primers produced a product of 590 bp; the B19 VP1-specific primers produced a product of 104 bp. β-Globin single-round PCR was performed using the same reagents and parameters. β-Globin–specific primers have been described previously (Taller *et al*, 1996) and produce a 437-bp product. PCR products were analyzed by ethidium bromide (EtBr)-stained gels.

Every precaution was taken to minimize contamination. PCR reactions were prepared in a room separate from the analysis area. Benchtops, floors, and reusables were routinely treated with 10% bleach prior to use. No wild-type AAV2 or B19 work had ever been performed in the laboratory at the time of this investigation.

All samples were tested two to seven times for AAV2, two to five times for B19 VP1, and one to two times for B19 NS1. Only those nPCR reactions that produced a strong (intense) band were considered positive. For a sample to be considered positive, each nPCR result had to be positive (for B19, both VP1 and NS1 had to be positive) each time the sample was tested. If every nPCR result was negative, the sample was determined to be negative. Any samples with any positive results but less than 100% were deemed equivocal. The range for a given group (as in Table 1) was determined by adding the equivocal percentage to the positive percentage.

Statistical analysis
Chi-square analyses were performed to determine the statistical significance of the differences found among the three subgroups of the study population and whether there was any gender effect. A P value of .05 was adopted as the threshold for assigning statistical significance on all tests.

Sequencing of PCR products and BLAST
nPCR products were extracted from agarose gels using a kit (Qiagen) according to the manufacturer's instructions and were sequenced by the University of Florida ICBR DNA Sequencing Core facility using the flourescent dideoxy terminator method of cycle sequencing (McCombie *et al*, 1992; Smith *et al*, 1986) on

either a Perkin Elmer, Applied Biosystems Division (PE/ABd) 373A or 377 automated DNA sequencer, following ABd protocols. Sequencing was performed unblinded. Sequences obtained were compared to known parvoviral sequences using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST; Altschul *et al*, 1997) program (www.ncbi.nlm.nih.gov/BLAST).

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