

REVIEW **Borna disease virus**

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Borna disease virus (BDV) is unique amongst animal RNA viruses in its molecular biology and capacity to cause persistent, noncytolytic CNS-infection in a wide variety of host species. Unlike other non-segmented negative-strand RNA animal viruses, BDV replicates in the nucleus of the host cell where splicing is employed for expression of a very compact genome. Epidemiological studies indicate a broad host range and geographical distribution, and some investigators have proposed that human infection may result in neuropsychiatric disorders. Experimental Borna disease in neonatal and adult rats provides an intriguing model for immune-mediated disturbances of brain development and function. Copyright © 2001 John Wiley & Sons, Ltd.

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BACKGROUND

Borna disease (BD) is a severe, frequently fatal, immune-mediated meningoencephalitis that most commonly affects horses and sheep in central Europe. The causative agent, Borna disease virus, is a noncytolytic, enveloped neurotropic RNA virus. BD can be induced by experimental infection in a wide variety of animals including birds, rabbits and non-human primates, but is best characterised in rat models. The broad experimental host range has led to speculation that humans can also become infected with BDV.

Two rodent models of BDV infection are established: infection of immunocompetent, adult rats where animals have meningoencephalitis similar to that described in ungulates with BD and characteristic movement and behavioural disturbances; and infections of neonatal rats where subtle disturbances in learning and behaviour, and developmental neuropathology are found in the absence of overt meningoencephalitis. Recently, there has been increasing focus on this

latter model due to its potential to provide insights into human neuropsychiatric disorders such as autism and schizophrenia where perinatal pathology is proposed to be important.

TAXONOMY

BDV is the prototype of the family *Bornaviridae*, genus *Bornavirus*, within the non-segmented negative strand (NNS) RNA viruses (order *Mononegavirales*). At approximately 9.9 kb the genomic RNA of BDV is substantially smaller than those of other *Mononegavirales*, the *Rhabdoviridae* (approximately 11–15 kb), *Paramyxoviridae* (approximately 16 kb) and *Filoviridae* (approximately 19 kb). Complete BDV genomic sequences have been reported for two virus isolates, Strain V and He/80 [1,2], which are approximately 94% identical at the nucleotide level. BDV is characterised by extraordinary sequence conservation uncommon for RNA viruses, where the inherent low fidelity of viral RNA dependent RNA polymerases (RdRps) results in sequence divergence of 10^3 – 10^4 per site per round of replication [3]. Studies of two ORFs (nucleoprotein and phosphoprotein) from widely disparate BDV isolates revealed variability of up to 4.1% at the nucleotide level and 1.5% to 3% at the predicted amino acid level [4,5]. A new genotype, termed No/98, with 15% divergence at nucleotide level was recently isolated from a naturally infected horse in Austria [6].

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Abbreviations used:

5HT, serotonin; BD, Borna disease; BDV, Borna disease virus; CK II, casein kinase II; NE, norepinephrine; NLS, nuclear localisation sequence; NNS, non-segmented, negative strand; nt, nucleotide; PKC ϵ , protein kinase C ϵ ; RdRp, RNA-dependent RNA polymerase; TF, tissue factor; TGF, transforming growth factor

MOLECULAR BIOLOGY OF BDV

Genomic organisation

BDV encodes six ORFs in three transcription units framed by complementary termini similar to those of other NNS RNA viruses (Figure 1) [1,7]. The first transcription unit encodes only one protein, the nucleoprotein p40 (N). The second transcription unit encodes p10 (X) and the phosphoprotein

p24 (P) in overlapping ORFs. The third unit contains coding sequence for the atypical glycoprotein and putative matrix protein gp18 (M), type I membrane glycoprotein gp94 (G) and polymerase p190 (L).

Replication and transcription of the BDV genome occur in the cell nucleus. While nuclear replication and transcription are found in segmented negative-strand RNA viruses and nucleo-

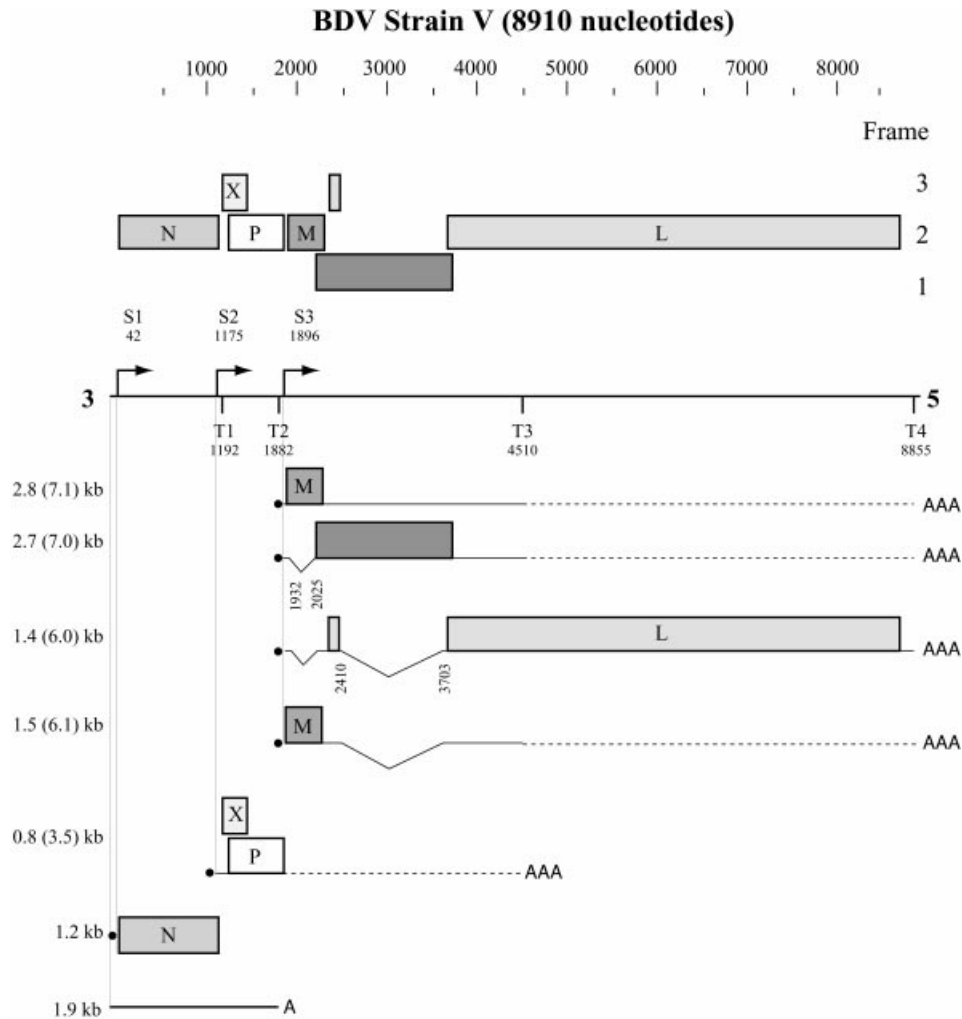


Figure 1. BDV strain V genomic map, transcripts and spliced products. N, nucleoprotein; X, protein X; P, phosphoprotein; M, putative matrix protein; G, glycoprotein; L, polymerase; S1–S3, initiation sites of transcription; T1–T4, termination sites of transcription; kb, 1000 bases. The genomic RNA is depicted in 3' to 5' orientation to reveal the six major ORFs. The sizes of mRNAs resulting from readthrough at termination signals T2 or T3 are in parenthesis, the corresponding readthrough RNA segments are indicated by dashed lines. Readthrough at T2 has been observed by northern blotting but only readthrough at T3 appears to be essential for viral gene expression. Note that the non-coding 1.9 kb RNA initiates at the genomic promoter, not at S1. The first and second transcription units overlap in the intergenic region. The genomic and antigenomic RNAs are unmodified at the termini; mRNA transcripts are capped at the 5' end (indicated by bold circles, ●) and polyadenylated at the 3' end; the 1.9 kb RNA is uncapped but can be partially polyadenylated. The positions of the transcription signals are given for the antigenomic (coding-orientation) RNA; numbers written in vertical orientation indicate positions of introns

rhabdoviruses (NNS RNA viruses of plants such as sonchus yellow net virus), BDV is the only known NNS RNA virus of animals with this property [8].

The 5' and 3' terminal untranscribed sequences of *Mononegavirales* typically encode promoters that serve for both replication and transcription of the genome. BDV contains 42 nontranscribed nucleotides at the 3' end of the genome, which presumably contain the promoter for expression of the viral positive-sense antigenome and viral transcripts, and 54 nucleotides at the 5' end for expression of the negative-sense genome during replication [9]. In other *Mononegavirales* the ratio or abundance of viral proteins during the replication cycle in the cell regulates the switch from transcription to production of full length viral antigenome and genome from the same promoter sequences rather than from different promoters. However, *in vitro* replication/transcription and reverse genetic systems are not yet established for BDV; thus, there are no direct data concerning how BDV regulates transcription and replication.

BDV mRNAs are capped and polyadenylated [1,2] (Figure 1). The first transcription unit (initiation of transcription at S1 and termination at T1) produces a single mRNA of 1.2 kb encoding N. The second initiation signal S2 is utilised for synthesis of the bicistronic 0.8 kb mRNA (termination at T2) and 3.5 kb mRNA (readthrough at T2 and termination at T3) encoding P and X. The third initiation signal S3 also produces two sets of mRNAs by alternative termination of transcription: the 2.8 kb mRNA (termination at T3) and 7.1 kb mRNA (readthrough at T3 and termination at T4) encoding M. Third transcription unit mRNAs can be processed by splicing of one or two introns to yield RNAs allowing translation of G (2.7 and 7.0 kb mRNA) or L (6.0 kb mRNA). The 1.9 kb RNA is fundamentally different from the other subgenomic transcripts. It starts at the 3' terminus of the genome rather than at the first transcription initiation signal, is not capped and only partially polyadenylated. Whether it represents an analogue of leader-containing subgenomic RNAs found in other NNS RNA viruses or an abortive replication intermediate with a stop at the second termination signal is unknown.

The molar abundance of the individual BDV

mRNAs in infected cultured cells and tissues [1,2] resembles the 3' to 5' declining transcriptional gradient of rhabdoviruses and paramyxoviruses [10]. NNS RNA viruses typically contain signal sequences in the noncoding intergenic regions of the genome that specify transcriptional termination/polyadenylation and initiation by the viral polymerase complexes [10]. RNA circularisation and RT-sequencing over the ligated ends revealed that BDV contains three semiconserved, uracil-rich initiation motifs, that are unique to BDV, and four stop signals that, similar to other *Mononegavirales*, contain an adenosine followed by six or seven uracil residues [9]. However, the gene junctions are atypical for *Mononegavirales* as they cannot be clearly divided into discrete regions corresponding to a termination signal for one transcription unit, an intergenic region and an initiation signal for the next transcription unit. The first and the second transcription units overlap such that the second initiation signal S2 lies upstream of the first termination signal T1 (Figure 1). A similar organisation is postulated to serve as an attenuation signal for control of polymerase expression in RSV [11]; however, in BDV, attenuation appears not to take place as the two transcription units are expressed at similar levels [1]. The second and third transcription units are separated by only two nucleotides, with the second termination signal, T2, fully contained within the third initiation signal S3. Initiation signal S3 gives rise to two different primary transcripts, the first terminating at the third termination signal T3, the second is expressed by readthrough of T3 and termination at T4 (Figure 1). Indeed, readthrough of T3 in BDV is essential for the expression of p190, the polymerase protein. Transcriptional readthrough may provide means for regulating expression of the BDV polymerase, a protein only needed in catalytic amounts. Consistent with this notion is the observation that levels of the 7.1 kb transcript are lower than those of the 2.8 kb transcript [1].

Splicing

Splicing in BDV allows for efficient use of its genome, controls expression of three ORFs comprising the third transcription unit, and may have implications for neurotropism and pathogenesis. Introns are located at nt 1932–2025 (intron-1) and nt 2410–3703 (intron-2) [12,13] (Figure 1). Differ-

ential splicing and potential for either termination or readthrough at the termination signal T3 allows for expression of six additional RNAs [12]. Differential splicing of the two introns regulates expression of the M, G and L proteins. Splicing of intron-1 places the thirteenth aa residue of the M ORF in frame with a stop codon. While this abrogates M expression, the resulting 13 aa minicistron facilitates G expression by ribosomal reinitiation [14]. Splicing of intron-2 fuses 17 nt of upstream sequence containing an AUG to a continuous ORF comprising the remainder of the L coding sequence (nt 3703–8819) [15].

Nucleoprotein

The first ORF of the BDV antigenome codes for a single protein. Although participation in RNP complex formation and binding to RNA has yet to be demonstrated, its position in the viral genome, size and relative abundance in infected cells suggest that it corresponds to the nucleoprotein (N).

N exists as either a 40 kDa or 38 kDa form. The p38 ORF starts at the second in frame AUG and lacks 13 aa at the amino terminus. The p38 ORF is predicted by Kozak's rules [16] to be in better context for translation initiation than the AUG corresponding to the p40 ORF [17]. It is unknown whether p38 and p40 are translated from a single transcript. Pyper *et al.* report the presence in infected cells of an RNA that initiates downstream of S1 and encodes p38 [17]. Immunohistochemical and cell fractionation experiments with cells transfected for expression of p40 or p38 revealed that whereas p40 is primarily nuclear, p38 is primarily cytoplasmic. This difference in the subcellular distribution of p40 and p38 reflects the presence of a nuclear localisation signal ($_3\text{PKRRLVDDA}_{11}$) in p40 [17,18]. The *in vivo* significance of the two isoforms of N is unknown. Both p38 and p40 bind the BDV phosphoprotein p24 (P). The phosphoprotein contains a potent nuclear localisation signal; thus, it is conceivable that the 38 kDa isoform may enter the nucleus through interaction with P.

X-Protein

Unlike the first transcription unit, the second transcription unit is bicistronic and encodes two polypeptides, p10 (X) and P. X is expressed from an ORF that initiates upstream of P and

overlaps with this ORF in a +1 frame shift [7]. Expression levels relative to N and P have not been established but X is readily detected in infected cells and tissues. X is a cytoplasmic protein when expressed in the absence of viral factors in transfected cells but localises to the nucleus in infected cells. This change in subcellular localisation appears to be mediated by direct interaction with P and indirect interaction with p40 [19–21], both of which possess NLS sequences. Interaction with viral proteins proposed to be involved in assembly of productive RNPs and coupling translation of X to P via a bicistronic mRNA suggests that X may play a role as cofactor for the viral RdRp. The amino terminus of X was recently shown to be critical for binding of P [21,22] and contains a leucine-rich region ($_2\text{SSDLRLTLELVRRLN}_{17}$) that is similar to nuclear export sequences of other cellular and viral export proteins including HIV-1 Rev, HSV ICP 27, and PKI [23]. X may therefore also be involved in nucleocytoplasmic shuttling of RNPs and/or subgenomic BDV transcripts in a fashion that may be modulated by binding of P.

Phosphoprotein

P is expressed from the second ORF of the second transcription unit of the BDV genome. There is no evidence of splicing to eliminate the first AUG initiating translation of X [1,24]; thus, it is likely that P is expressed through a leaky scanning mechanism. The phosphoproteins of NNS RNA viruses are essential cofactors for virus transcription and replication. Their phosphorylation by cellular kinases influences the ability of phosphoproteins to form homomultimers, bind other viral proteins and serve as transcriptional activators. Although there are no direct data concerning the role of P in the BDV lifecycle, it is postulated to have a similar function to other viral phosphoproteins. P contains a strong bipartite NLS at its amino-terminus and appears to have weaker NLS motifs toward its carboxyl terminus [25]. Mammalian two-hybrid and coimmunoprecipitation experiments indicate that P interacts with itself, X and N. Analysis of P truncation mutants led to identification of three nonoverlapping regions important for oligomerisation (aa 135–172), binding to X (aa 33–115) and N (aa 197–201) [20]. P also colocalises with N and X in distinct areas within the nuclei of infected cells [7].

Investigation of the phosphorylation sites of P revealed a complex phosphorylation pattern. P is phosphorylated predominantly by protein kinase C ϵ (PKC ϵ) and, to a lesser extent, by casein kinase II (CK II). Peptide mapping studies identified Ser₂₆ and Ser₂₈ as sites for PKC ϵ , and Ser₇₀ and Ser₈₆ as sites for CK II phosphorylation [26]. In other viral systems, sequential phosphorylation by different kinases may be associated with conformational changes that influence protein:protein interactions [27]. Whereas the PKC ϵ phosphorylation sites of P overlap with the NLS, the CK II phosphorylation sites overlap with its region of interaction with X. Thus, it is conceivable that the state of P phosphorylation may influence nuclear trafficking of P and X (through its interaction with P). PKC ϵ is highly concentrated in limbic circuitry [28]. This observation is intriguing given the limbic distribution of BDV, and suggests that phosphorylation events may be important in BDV neurotropism.

gp18

The first ORF of the third transcription unit predicts a protein of M_r 16 000; however biochemical analysis revealed that this protein is N-glycosylated with an M_r of 18 000 (gp18, M) [29-31]. Endoglycosidase sensitivity and lectin staining patterns suggest that M is likely to be a glycoprotein of the biantennary complex type. Mass spectrometric studies indicate that the protein forms stable tetramers *in vivo* and contains hydrophobic sequences characteristic for membrane spanning proteins [32]. Two observations suggest that M is expressed at the surface of viral particles and mediates the attachment of the virions to cellular receptors: (i) antisera against M have neutralising activity [33]; and (ii) incubation of susceptible cells with M prevents infection [31]. The role of M in the BDV life cycle remains to be determined. In nonsegmented, negative-strand RNA viruses, the third gene usually directs expression of a matrix protein. Matrix proteins in *Mononegavirales* are not known to be glycosylated; however, glycosylated matrix proteins that resemble M in size and isoelectric point of about 10 units have been found in other viral systems (for example, M (E1), in coronaviruses [34]). Further studies need to be done to verify whether M is an unusual matrix protein or a novel viral cofactor surface protein mediating attachment.

Glycoprotein

The second ORF of the third transcription unit predicts a protein with an M_r of 57 000. Post-translational modification by N-glycosylation with high-mannose and/or hybrid oligosaccharides yields a full length type I membrane protein of M_r 94 000 (G) [35,36] that is subsequently cleaved by the cellular protease furin [35]. The levels of G *in vitro* and *in vivo* are likely to be lower than those of other structural proteins. Additionally, there are data to suggest that it may not be expressed in all infected cells [35]. A glycosylated M_r 43 000 carboxyl-terminal cleavage product has been found in infected cells; an amino-terminal cleavage product is inferred but not confirmed. Although G may be expressed from unspliced transcripts by leaky ribosomal scanning, intron-1 spliced transcripts are more likely to serve as messages for G *in vivo*. Splicing of intron 1 results in a 13 aa minicistron that enhances the translation the G-ORF presumably by facilitating ribosomal reinitiation [14].

Both full length protein (M_r 94 000) and the C-terminal cleavage product (gp43) are incorporated into virions; only gp43 is translocated to the cellular membrane [37]. G and/or gp43 are likely to mediate early events in infection such as attachment and/or entry. The activity of neutralising sera from infected animals is reduced following immunoadsorption with the G-protein [36]. Furthermore, preincubation of susceptible cells with G interferes with infectivity [36]. Cleavage of G appears to be a prerequisite for infectivity [35].

Polymerase

By position and its large size that spans almost 60% of the genome the last ORF is predicted to encode the viral RdRp (L) [1,24]. Expression of this ORF from the first AUG conserved between strains V and He/80 would yield a protein of M_r 170 000 and result in approximately 400 bases of non-coding region between the end of G and initiation of L. However, full sequence conservation between the two strains upstream of the first conserved AUG suggests that this region also is expressed. Splicing of mRNAs originating from the third transcription unit provides a mechanism for translation of this upstream region by fusion to an AUG contained in a small ORF that overlaps with the 5'-end of the gene for G. The presence of

this additional coding sequence in the L mRNA increases the predicted M_r for the polymerase to 190 000 and was recently demonstrated using antibodies directed against this amino-terminal fusion portion of L [15]. Overexpression of recombinant L also confirmed interaction with P and indicated that L is phosphorylated by cellular kinases [15].

HOSTS AND EPIDEMIOLOGY

A syndrome of progressive meningoencephalitis of horses and sheep consistent with BDV infection [38] was recognised 100 years before the disease received its name from an equine outbreak in 1895/96 near the town Borna in Germany. Although this clinical pattern is still considered to represent classical BD, infection may also result in asymptomatic carrier status, subtle disturbances in learning and memory, profound disorders of behaviour and movement [39-42], or death. Accumulating epidemiological data, including reports of asymptomatic, naturally infected animals [43-49], suggest that the host range and geographical distribution of BDV are larger than previously appreciated. Natural infection has been reported in a wide variety of hosts including horses, donkeys, sheep, cattle, dogs, cats, rabbits and birds. Experimental infection has been achieved in many of these species and also in rodents and primates. Whether BDV naturally infects humans remains controversial; however, there is consensus that all warmblooded animals are likely to be susceptible to infection. Although Central Europe has the highest reported prevalence of Borna disease, natural infection has been described throughout Europe, in Asia and in North America. It is unclear whether the apparent increase in host and geographical range of BDV is due to spread of the virus or simply enhanced awareness of the agent and improved diagnostic reagents.

Neither the reservoir nor the mode for transmission of natural infection are known. Natural infection of horses and sheep is typically sporadic and peaks in spring months; epidemics of disease are infrequent (reviewed in [50]). An olfactory route for transmission has been proposed because intranasal infection is efficient and the olfactory bulbs of naturally infected horses show inflammation and oedema early in the course of disease [51,52]. One outbreak in a zoo has been attributed

to inadvertent inoculation of BDV during vaccine administration (Müller H, personal communication). Experimental infection of neonatal rats results in virus persistence and is associated with the presence of viral gene products in saliva, urine and faeces [53]. Such secreta/excreta are known to be important in transmission of other pathogenic viruses (for example, lymphocytic choriomeningitis virus, hantaviruses). Normal adult rats housed in cages separate but adjacent to those of neonatally infected rats can become infected suggesting the possibility of transmission by aerosol or fomites (Hornig M, Solbrig MV and Briese T, personal communication). Reports of BDV nucleic acid and proteins in PBMC also indicate a potential for haematogenous transmission [53,54]. The observations that rodents can be persistently infected with BDV and excrete virus suggest that they have the potential to serve both as natural reservoirs and vectors for virus dissemination. However, the only study of BDV infection in wild rodents did not indicate natural infection [55]; thus, the significance of rodents for transmission of BDV to other domesticated animals and humans remains speculative. Recently, vertical transmission of BDV has been demonstrated in a pregnant mare [56].

NATURAL INFECTIONS

Although infection may be subclinical, symptomatic disease typically follows a predictable course. Clinical signs at the onset of disease in horses and sheep are nonspecific and include: excited or depressed behaviour, hyperthermia, anorexia, jaundice, constipation and colic [42]. Classical disease becomes apparent within 1-2 weeks. Animals maintain an upright, wide-based stance with their heads extended. Repetitive behaviours are common and may include vacuous chewing, circular ambulation and running into obstacles. Horses become paretic in the terminal phases of disease. A distinctive decubitus posture associated with paddling movements of the legs has been described. Frequently, in late disease the virus migrates centrifugally along the optic nerve to cause retinopathy and visual impairment. Acute mortality may be as high as 80%-100% in horses and 50% in sheep [42]. Sheep that survive may have permanent neurological deficits. Recurrence of acute disease has been described in sheep [57]. Natural symptomatic infection with fatal

outcome has also been reported in cattle [58], rabbits [59], and one dog [60]. Epidemics of paresis in ostriches were attributed to BDV infection by serology [61]; however, these data have not been confirmed by other methods. Neurological disease in cats in Sweden [62], Japan [63] and Great Britain [64] has been attributed to BDV infection using serology and molecular methods. BDV infection was found in cats with a specific syndrome, staggering disease, in Sweden [65,66] but not in Austria [67].

Viral persistence without apparent disease has been described in naturally infected horses [43-45], sheep [46,47] and cats [48] in Europe and Asia. There is one report indicating subclinical infection of horses in North America [49].

EXPERIMENTAL MODELS OF BORNAVIRUS INFECTION

A wide range of animal species have been experimentally infected with BDV. Rats [39], mice [68-70], hamsters [71], Mongolian gerbils [72], rabbits [73-75], guinea pigs [73], tree shrews [76], rhesus monkeys [77-81] and chickens [82] are all susceptible to classical disease; however, the incubation period, mortality and severity of disease varies considerably between species, strains within a species, and immune status of the host. Whereas in adult immunocompetent hosts infection results in dramatic immune-mediated meningoencephalitis consistent with the classical syndrome observed in naturally infected horses and sheep, animals less able to respond to infection due to immature or compromised immune systems have a more subtle course.

Adult rat model

Susceptibility to disease varies with the host rat strains. Wistar rats and black-hooded (BH) rats can be productively infected but have less severe disease than Lewis rats, a strain with a lesion in the hypothalamic-pituitary adrenal axis associated with enhanced susceptibility to immune-mediated disorders such as experimental allergic encephalomyelitis and adjuvant induced arthritis [83,84]. One report found that resistance to BD was inherited as a dominant trait in Lewis and BH hybrids that was independent of MHC genes [85]. Virulence of viral strains for rats may be enhanced by serial passages of virus in rat brain [86].

Infection is most rapidly achieved with intra-

cranial and intranasal inoculations. Nonetheless, any route of inoculation that allows virus access to nerve terminals (for example, intramuscular, intraperitoneal or footpad injection) ultimately results in central nervous system infection and classical disease. Viraemia is unlikely to play a significant role in BDV dissemination and pathogenesis. Although viral gene products have been found in PBMC of infected animals [87] intravascular inoculation only infrequently results in productive infection. Several observations indicate that BDV disseminates primarily via neural networks: (i) viral proteins and nucleic acids can be traced centripetally and transsynaptically after olfactory, ophthalmic or intraperitoneal inoculation [88,89]; (ii) the onset of disease is delayed in parallel with distance from the inoculation site to the central nervous system [89]; and (iii) migration of virus to the CNS after footpad infection can be prevented by sciatic nerve transection [89]. Irrespective of the route of inoculation, the onset of clinical disease coincides with appearance of viral proteins in hippocampal neurons and the onset of meningitis [89,90]. It has been proposed that BDV, like rabies virus, is likely to spread as an RNP complex within neural networks [91]. Although structures consistent with RNPs have been described in neurons of experimentally infected animals [81,92] the form of disseminating virus is unknown.

The immune-mediated disorder in adult infected rats presents clinically as hyperactivity and exaggerated startle responses 10-14 days after intracerebral infection [93]. The acute phase coincides with infiltration of monocytes into the brain, particularly in areas of high viral burden including the hippocampus, amygdala and other limbic structures [89] (Figure 2B). Two to three weeks later, rats show high grade stereotyped motor behaviours (the continuous repetition of behavioural elements, including sniffing, chewing, scratching, grooming and self-biting), dyskinesias, dystonia and flexed seated postures [94]. Five to ten percent of animals become obese, achieving body weights up to 300% of normal rats [52]. Some investigators have reported isolation of a BDV strain that causes obesity in more than 50% of adult infected rats [95].

Disorders of movement and behaviour are linked to distinct changes in CNS dopamine systems [94,96,99]. Preliminary data also suggest

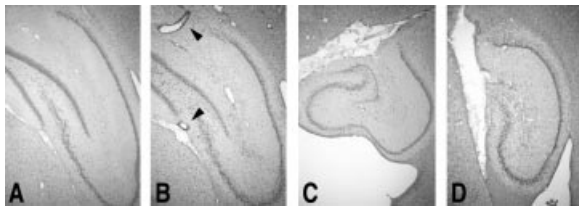


Figure 2. Hippocampal pathology in adult and neonatally infected Lewis rats. Dentate gyrus in an uninfected rat (A); adult infected rat 6 weeks post infection in the acute phase of disease (B); adult infected rat 5 months post infection in the chronic phase of disease (C); and neonatal infected rat 6 months post infection (D). Arrows in B indicate immune cell infiltrates in perivascular distribution. Note loss of cells and structural integrity in late phases of the disease (C). The neonatal infected rat is characterised by disturbances in the architecture of dentate gyrus without robust inflammatory cell infiltrates (D). Haematoxylin and eosin staining, original magnification $\times 25$

abnormalities of serotonin (5HT) metabolism [96]. Adult-infected BD animals are more sensitive to dopamine agonists and antagonists than normal rats. Administration of the indirect dopamine agonist dextroamphetamine to infected animals results in increased locomotor and stereotypic behaviours [94]. Similarly, cocaine-mediated inhibition of dopamine reuptake potentiates dopamine neurotransmission resulting in enhanced locomotion and stereotypic behaviours [99]. The movement and behaviour disorder is improved following treatment with selective dopamine antagonists; whereas D2-selective antagonists (for example, raclopride) do not affect locomotor responses in BD rats, high doses of selective D1 antagonists (for example, SCH23390) and atypical dopamine blocking agents with mixed D1 and D2 antagonist activity (such as clozapine) selectively reduce locomotor activity in BD rats but not in controls [94].

The basis for these functional disturbances appears to be partial dopamine deafferentation with compensatory metabolic hyperactivity in nigrostriatal and mesolimbic dopamine systems. At the receptor level, both pre- and postsynaptic sites of the dopamine transmitter system appear to be damaged in striatum (caudate-putamen and nucleus accumbens); dopamine reuptake sites are reduced in nucleus accumbens [98] and caudate-putamen [99]; D2 (but not D1) receptor binding is markedly reduced in caudate-putamen; D2 and D3 receptor binding are reduced in nucleus accumbens [94,97,98]. It is possible that the

differential effects of infection on dopamine receptors in nucleus accumbens and caudate-putamen may reflect BDV-mediated interference with the cellular transcription and/or splicing machinery. Whereas binding to receptors expressed from spliced messages, D2 and D3, is reduced, binding to D1, a receptor expressed from an unspliced message, is not.

Although the increased locomotor activity, stereotypic behaviours and dyskinesias of the adult BD model are linked to distinct disturbances in dopamine pathways, additional neuromodulator abnormalities have also been noted. The expression of genes for neuromodulatory substances and their associated synthesising enzymes, including somatostatin, cholecystokinin and glutamic acid decarboxylase, is greatly reduced during the acute phase and recovers toward normal in the chronic phase of adult BD [100]. The cholinergic system, a major component in sensorimotor processing, learning and memory, also appears to be affected in adult BDV infection. A progressive decrease in the number of choline acetyltransferase-positive fibres in hippocampus and neocortex has been observed to begin as early as day 6 postinfection [101]. Preliminary work on dysregulation of the 5HT and norepinephrine (NE) systems suggests metabolic hyperactivity of 5HT. There is a modest increase in the 5HT metabolite 5-hydroxyindoleacetic acid in the striatum and of the NE metabolite 3-methoxy-4-hydroxyphenethyleneglycol in the prefrontal and anterior cingulate cortex [96]. These changes may reflect compensatory upregulation following partial loss of dopamine afferents to these brain regions. Selective effects of BDV on 5HT and NE pre- or postsynaptic receptors have not yet been investigated. The mechanism by which adult BD rats are rendered hypersensitive to the hyperkinetic and convulsant effects of the opiate antagonist, naloxone [102], is also unclear. In addition, because adult infected rats have marked CNS inflammation, it has not been possible to determine whether monoamine, cholinergic and opiate-tergic dysfunction in BD results from direct effects of the virus, virus effects on resident cells of the CNS, or a cellular immune response to viral gene products.

In late disease, BDV disseminates throughout the autonomic and peripheral nervous systems and can be readily detected in autonomic plexi in

the lungs and gastrointestinal tract and at the neuromuscular junction [89,103]. It is also present at lower levels in non-neural tissues including bone marrow, thymus and PBMC [53,54]. Although numbers of mononuclear inflammatory cells in the CNS are markedly reduced during the chronic phase of disease, there is an elevation in titres of antibodies directed against all BDV proteins (N, X, P, M, G and L). Antibodies specific for M [33] and G have neutralising activity *in vitro* [36,37]. Although virus is not cleared from the brain, neutralising antibodies may modulate viral gene expression [104] and limit the infection to the CNS, preventing further dissemination to non-neural organs [103]. The blood-brain barrier remains functional in various assays [104–106]. The life expectancy of animals that have progressed into the chronic phase may be normal, although loss of parenchymal brain tissue, low during the acute phase, continues at an increased rate and can reduce brain mass by up to 50% [93,107] (Figure 2C).

Neonatal rat model

Neonatally infected rats display a wide range of physiological and neurobehavioural disturbances. They are smaller than uninfected littermates [108,109], have a heightened taste preference for salt solutions, and altered sleep-wake cycles [108]. The basis for runting is unknown: there is no apparent alteration in levels of glucose, growth hormone, or insulin-like growth factor-1 [108] or amount of food ingested [110]. Behavioural disturbances are less dramatic in neonatally infected animals than in their adult-infected counterparts. A study of behavioural and cognitive changes in Wistar rats infected in the neonatal period found spatial and aversive learning deficits, increased motor activity and decreased anxiety responses [111]. Similar deficits in spatial learning and memory were found by Carbone and colleagues in neonatally infected Lewis rats [108]. More recently, play behaviour has been reported to be abnormal in neonatal infected rats, with decreases in both initiation of nondominance-related play interactions and response to initiation of play by noninfected, age-matched control animals or infected littermates [112].

The neonatal infection model has not been studied as extensively as the adult infection model. CNS dysfunction in neonatally infected

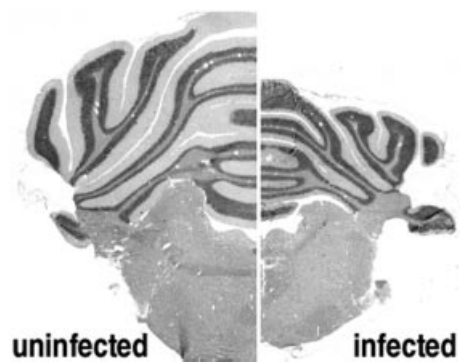


Figure 3. Cerebellar pathology in neonatally infected Lewis rats 12 weeks post infection. Coronal section of the cerebellum and brainstem of an uninfected (left) and age-matched, neonatally infected (right) rat demonstrating cell loss in the cerebellar molecular layer but preservation of cerebellar foliation. Haematoxylin and eosin staining, original magnification $\times 3$

animals has been proposed to be linked to viral effects on morphogenesis of the hippocampus (Figure 2D) and cerebellum (Figure 3), two structures in rodents that continue to develop after birth [113–116]. Carbone and colleagues found a quantitative relationship of hippocampal pathology to behavioural abnormalities in the neonatal infection model; the extent of neuronal loss in the dentate gyrus appeared to be correlated with the severity of spatial learning and memory deficiencies in neonatally infected Lewis rats [108].

Humoral immune responses to BDV in neonatally infected animals are significantly lower than in adult infected animals [109]. There is a transient, nonspecific cellular immune response that peaks approximately 3 weeks post intracerebral infection and dissipates within 10–14 days [117,118]. This period coincides with the presence of high levels of mRNAs for cytokine products of CNS macrophages/microglia (IL-1 α , IL-1 β , IL6 and TNF- α) in hippocampus, amygdala, cerebellum, prefrontal cortex and nucleus accumbens [117,118]. The role of the extrinsic immune response is unclear as thymectomised animals show similar behavioural disturbances and brain pathology in the absence of infiltrating inflammatory cells (unpublished results).

Marked astrocytosis has been noted in dentate gyrus and cerebellum [109,110,119]. Upregulation of tissue factor (TF) has been identified as one mechanism by which BDV may alter CNS development [119]. TF is a member of the class II cytokine receptor family primarily produced by

astrocytes that plays important roles in cellular signal transduction, brain function and neural development through its effects on coagulation protease cascades. However, cerebellar changes cannot be explained by this mechanism, as TF upregulation is not observed in the cerebellum despite prominent astrocytosis. Furthermore, BDV infection of astrocytes appears to be required for TF upregulation [119], and cerebellar astrocytes are reported to be spared from BDV infection, at least for 30 days following neonatal infection [110].

Behavioural disturbances (such as stereotypies, inhibited responses to novel stimuli and impaired motor development) and neuropathology (such as Purkinje and granule cell loss in cerebellum) of neonatal Borna disease [112,117] overlap with the clinical aspects of autism [120-122]. Although BDV infection has not been detected in children with autism these similarities in pathology suggest that the neonatal Borna disease model may be useful for investigation of dysregulation of developmental programmes due to perturbations in the expression of soluble factors such as cytokines and neutrophins.

Mouse model

Mice are readily infected and have high titres of the virus in the brain [69]. Until recently mice were considered to be relatively resistant to disease; however, two reports indicate that disease can be induced by adaptation of virus through multiple passages in mice [70] or infection of specific host strains (MRL mice) during the neonatal period [68]. As in rats, severe clinical disease is mediated by MHC class I restricted cytotoxic T-cells [68].

Prosimians and non-human primates

Little is known about BDV pathogenesis in phylogenetically higher species such as non-human primates and the prosimian tree shrews (*Tupaia glis*). Intracerebral inoculation of tree shrews leads to persistent infections and a disorder characterised primarily by hyperactivity and alterations in sociosexual behaviour rather than motor dysfunction [76]. Disturbances in breeding and social behaviour were most profound in animals caged in mating pairs. Females, rather than males initiated mating, and infected animals failed to reproduce despite increased

sexual activity. Although detailed neuroanatomical studies were not performed, the syndrome was interpreted to be due to neuropathological changes in the limbic system.

The only reported studies of experimentally infected primates employed adult Rhesus macaques (*Macaca mulatta*). These animals were initially hyperactive and subsequently became apathetic and hypokinetic. Pathology was remarkable for meningoencephalitis and retinopathy [80].

IMMUNE RESPONSE AND BDV PERSISTENCE IN THE CNS

Neurotropism affords BDV an opportunity to persist in an environment characterised by a restricted immune response. However, this does not reflect a failure to induce an immune response. Indeed, humoral immunity appears responsible for confining infection to the CNS [103] while cellular immunity is essential for expression of classical disease. Early studies of BD reported a correlation between the severity of meningoencephalitis and the clinical manifestations of disease [123]. The first direct evidence for the role of the immune response in the pathogenesis of BD emerged from studies in which splenectomised rhesus monkeys became persistently infected but had prolonged incubation periods and less severe disease compared with immune-competent animals [80,81]. Experiments with infected athymic rats or adult rats immunosuppressed by treatment with cyclophosphamide and cyclosporin A [93,124,126] provided further evidence that BD is immune-mediated.

In acute BD, CNS infiltrates are comprised of macrophages, CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, NK cells and to a lesser extent, plasma cells [106,127]. Antibodies to CD4 and CD8 markers were initially used to deplete these cells and investigate their individual contributions to immunopathogenesis. Although depletion of either CD4⁺ or CD8⁺ T lymphocytes ameliorated the inflammatory response in the brain and the severity of acute clinical disease [128,129], only depletion of CD8⁺ cells was found to reduce brain atrophy [130]. While these data may suggest that CD4⁺ T lymphocytes are not important in BD pathogenesis, more recent studies demonstrated that the recruitment of CD8⁺ cells is dependent on CD4⁺ T lymphocytes [131]. It has been argued that neuronal cells express little or no MHC class I

surface markers and should not be susceptible to CD8⁺ CTL lysis [132]; however, both neurons and astrocytes in BDV infected rats have been shown to express MHC class I protein [130,133]. Furthermore, lymphocytes isolated from BD rat brain were found to lyse infected target cells in an MHC class I-restricted manner [133]. Adoptive transfer experiments have been pursued with N protein-specific, CD4⁺ T lymphocytes. Immunosuppressed persistently infected rats so treated had a neurological disease that was similar, but not identical, to BD [90].

In animals that progress to chronic BD there is a pronounced decrease in CNS infiltration. The numbers of macrophages, CD4⁺ T lymphocytes and CD8⁺ T lymphocytes in brain are markedly reduced; however, the number of plasma cells are increased. In contrast to the immunopathogenesis of acute BD which has been studied extensively by several investigators, little is known about the basis for the decline in CNS infiltration in chronic infection. Potential mechanisms include altered viral gene expression or modulation of the immune response. The first possibility is unlikely because viral titres do not change substantively over the course of disease [93]. In addition, brain levels of N and P, the only BDV proteins which can be readily quantitated, and of RNAs coding for M, G and L [134] are similar in early and late disease. There is, however, increasing evidence for modulation of the immune response over the course of BD. Recent findings indicate the induction of BDV-specific type 1 T lymphocyte (Th₁) tolerance in chronic infection. Whereas lymphocytes isolated from brains of acutely infected rats have potent cytolytic activity, lymphocytes from brains of chronically infected rats do not lyse BDV-infected target cells [135]. Induction of BDV-specific tolerance in chronic infection may reflect the timecourse for presentation of viral antigens in the thymus [54]. Alternatively, Th₁ cells may become anergic or undergo apoptosis due to presentation of BDV antigens in brain without essential costimulatory signals [136-138]. Support for this hypothesis is found in the observation that apoptosis of perivascular inflammatory cells is most apparent at 5-6 weeks post infection, coincident with the onset of decline in encephalitis [139].

Variability in cytokine expression in brain over the course of BD may also be important for

modulation of the immune response. Cytokine mRNA levels were measured in rat brain at different times post-infection by RT-PCR [140], RNase protection assay or northern hybridisation [139]. IFN γ , TNF α , IL1 α and IL6 mRNAs are transiently expressed in the acute phase of the disease [139,140]. The message for another pro-inflammatory cytokine, IL2, was detected by RT-PCR experiments throughout infection [140]; however, RNase protection assays revealed that IL2 mRNA peaks in the acute phase of infection and declines thereafter [139]. IL2 and IFN γ are produced by Th₁ cells for recruitment and activation of CTL and stimulation of antigen presenting cells [141]. The peak in CNS levels of these proinflammatory cytokine mRNAs in acute BD is temporally correlated with the peak of the immune cell infiltration. Cytokines produced by type 2 T lymphocytes (Th₂), such as IL4, have the potential to downregulate the cellular immune response. IL4 mRNA levels are elevated throughout the course of BD, but increased in the chronic phase of infection [139]. IL10 mRNA, as measured by semiquantitative RT-PCR, peaks in the acute phase of BD and declines thereafter [139]. As in experimental allergic encephalomyelitis [142], the peak in IL10 mRNA expression is correlated with a shift away from CNS recruitment of immune cells. The high levels of IL4, IL6 and IL10 mRNAs observed in acute BD are likely to contribute to B cell activation. Indeed, in rats, progression toward the chronic phase of disease is accompanied by an increase in brain levels of BDV-specific antibodies [139].

The cytokine profile in chronic BD is consistent with modulation of the CNS immune response to reduce Th₁ T cell activation and increase the Th₂ humoral response. TGF β mRNA expression is elevated in BD-rat brain from the onset of acute disease [139] and its presence in chronic BD is compatible with a Th₂ response. TGF β has potential to mediate inhibition of the pro-inflammatory effects of TNF α , suppression of T and B cell growth, reduction of free radical formation and inhibition of antibody production and CTL activity [143,144]. Experimental administration of TGF β to BD-rats reduced the severity of encephalitis but did not prevent the onset of acute disease; thus, the role of TGF β in BD pathogenesis remains unclear [145].

The basis for the Th₁ to Th₂ switch in BD is a

matter only for speculation. There are no data to indicate whether presentation of antigen on MHC class I is insufficient or activation of the Th₁ cells is inefficient due to inadequate costimulation. How-

ever, the result of this switch is consistent with induction of Th₁ down-regulation, an event that would allow survival of the host and foster viral persistence.

Table 1. Serum immunoreactivity to BDV in subjects with various diseases

Disease	Prevalence		Assay	Reference
	Disease	Control		
Psychiatric (various)	0.6% (4/694)	0% (0/200)	IFA	[154]
	2% (13/642)	2% (11/540)	IFA	[155]
	4–7% (200–350/5000)	1% (10/1000)	WB/IFA	[156]
	12% (6/49)		IFA	[157]
	30% (18/60)		WB	[158]
	14% (18/132)	1.5% (3/203)	WB	[159]
	24% (13/55)	11% (4/36)	IFA	[160]
	0% (0/44)	0% (0/70)	IFA/WB	[161]
Affective disorders	4.5% (12/265)	0% (0/105)	IFA	[162]
	4% (12/285)	0% (0/200)	IFA	[154]
	38% or 12% (53 or 17/138)	16% or 4% (19 or 5/117)	WB (N or P)	[163]
	37% (10/27)		IFA	[157]
	12% (6/52)	1.5% (3/203)	WB	[159]
	0%–0.8% (0–1/122)	0% (0/70)	IFA/WB	[161]
	3.6% (9/251)	1.1% (10/917)	ECIA	[164]
	6.3% (2/32)	0% (0/28)		[152]
Major depression	25% (1/4)		IFA	[157]
	9%–28% (8 or 25/90)	0%–20% (0 or 4/20)	WB (N or P)	[165]
	17% (15/90)	15% (3/20)	IFA	[165]
	14% (16/114)	1.5% (3/203)	WB	[159]
	20% (2/10)		WB	[166]
	0%–1% (0–2/167)	0% (0/70)	IFA/WB	[161]
	3% (26/845)	1.1% (10/917)	ECIA	[164]
	24% (6/25)		WB	[167]
CFS	0% (0/75)	1.1% (10/917)	ECIA	[164]
	0% (0/169)	0% (0/62)	ELISA/WB	[168]
	100% (7/7)			[169]
	13% (15/114)	2.3% (11/483)	IP/IFA	[170]
MS	0% (0/50)		IFA	[171]
	7.8% (36/460)	2.0% (11/540)	IFA	[155]
HIV positive	8.1% (61/751)	2.3% (11/483)	IP/IFA	[170]
	0% (0/85)	1.1% (10/917)	ECIA	[164]
	9.8% (19/193)	2.3% (11/483)	IP/IFA	[170]
Schisto/malaria				

IFA, immunofluorescence assay; WB, western immunoblot; ECIA, electrochemiluminescence immunoassay; IP, immunoprecipitation; CFS, chronic fatigue syndrome; MS, multiple sclerosis; LAP, lymphadenopathy; Schisto/malaria, schistosomiasis or malaria; N, nucleoprotein; P, phosphoprotein.

Table 2. BDV nucleic acid in subjects with various diseases

Disease	Tissue	Prevalence		Divergence ^a	Reference
		Disease	Controls		
Psychiatric (various)	PBMC	67% (4/6)	0% (0/10)	0%–3.6%	[172]
	PBMC	37% (22/60)			[158]
	PBMC	42% (5/12)	0% (0/23)	0%–4.0%	[159]
	PBMC ^b	9% (3/33)	0% (0/5)		0.07%–0.83%
	PBMC	2% (2/106)	0% (0/12)		[161]
	PBMC	0% (0/24)	0% (0/4)		[166]
Affective disorders	PBMC	33% (1/3)	0% (0/23)		[159]
	PBMC	17% (1/6)	0% (0/36)		[160]
	Brain	40% (2/5)	0% (0/10)		[174]
	PBMC	4% (2/49)	2% (2/84)	0%–5.1%	[175]
	PBMC	0% (0/33)			[176]
	Brain	0% (0/11)			[177]
Schizophrenia	Brain	0% (0/3)	0% (0/3)		[178]
	CSF	0% (0/8)	0% (0/8)		[178]
	PBMC	0% (0/7)	0% (0/7)		[178]
	PBMC	64% (7/11)	0% (0/23)		[159]
	PBMC	10% (5/49)	0% (0/36)		[160]
	PBMC	100% (3/3)		4.2%–9.3%	[179]
	PBMC	0% (0/10)	0% (0/10)		[166]
	Brain	53% (9/17)	0% (0/10)		[174]
	PBMC	4% (3/77)	2% (2/84)	0%–5.1%	[175]
	PBMC	13.5% (10/74)	1.4% (1/69)		[180]
	PBMC	0% (0/39)			[176]
	Brain	0% (0/13)			[177]
CFS	PBMC	12% (7/57)		6.0%–14%	[181]
	PBMC	12% (3/25)			[167]
	PBMC	0% (0/18)			[168]
	PBMC	1/1		3.8%	[182]
Hippocampal sclerosis	Brain	80% (4/5)			[173,177]
Normal controls	PBMC		5% (8/172)		[183]
	Brain		6.7% (2/30)		[184]

CFS, chronic fatigue syndrome. ^a Divergence of P-gene nucleotide sequence from common BDV isolates (strain V and He/80). ^b PBMC-coculture.

BDV AND HUMAN DISEASE

Recognition of BDV's broad experimental host range and the observation that disturbances in behaviours in experimentally infected animals are reminiscent of some aspects of human neuropsychiatric diseases including major depressive disorder, bipolar disorder, schizophrenia and autism, led to the proposal that BDV might be implicated in their pathogenesis. Although there is consensus that humans are likely to be susceptible to BDV infection, the epidemiology and clinical

consequences of human infection remain controversial. There have been no large controlled prevalence studies. Furthermore, methods for diagnosis of human infection are not standardised; thus, it is difficult to pursue meta-analysis.

Epidemiological studies are complicated by a combination of properties of BDV replication: (i) The humoral immune response is often weak and assays for detection of antibodies specific for viral antigens (indirect immunofluorescence with infected cells; western immunoblot or EIA with

extracts of infected cells or recombinant proteins) are sometimes performed close to background levels. (ii) Even in the acute phase of infection BDV replicates to low titres [91] and may not be uniformly distributed throughout the brain [91,146]. These factors may confound the recovery of virus isolates and the detection of BDV gene products by *in situ* hybridisation or histochemistry. (iii) Detection of BDV in the periphery is also difficult. Neutralising antibodies confine BDV to the CNS [103]; however, BDV gene products have been detected in PBMC of infected ungulates and rodents and are reported by some investigators in either mononuclear cells or an as yet undefined cell that purifies with granulocytes in Ficoll preparations [147]. Those investigators who report detection of BDV nucleic acids in blood cells have employed nested RT-PCR, a method that is exquisitely sensitive to contamination artifact. Unfortunately, amplification products representing *bona fide* isolates cannot be readily distinguished from laboratory strains by sequence analysis because BDV is characterised by extraordinary sequence conservation [4,5]. Thus, similarities in sequence between putative new isolates and confirmed isolates cannot be used to exclude the former as artifacts. The extent to which sequence conservation in BDV represents enhanced polymerase fidelity, or more likely, selective environmental pressures is unknown.

Most reports suggesting an association between BDV and human disease have focused on neuropsychiatric disorders including unipolar depression, bipolar disorder, or schizophrenia; however, BDV has also been linked to chronic fatigue syndrome, AIDS encephalopathy, multiple sclerosis, motor neuron disease, panic disorders and brain tumours (glioblastoma multiforme) (Tables 1 and 2). The improbably broad spectrum of candidate disorders has led some investigators to propose that infection is ubiquitous and that in some disorders, elevation of serum antibody titres or the presence of viral transcripts in PBMC or neural tissues reflects generalised (AIDS) or localised (glioblastoma multiforme) immunosuppression.

There are only infrequent reports where infectious virus has been isolated from humans [147-150] or in which BDV gene products were found in human brain by *in situ* hybridisation and immunohistochemistry [150-152]. Phylogenetic

analysis of some reported sequences from human isolates is consistent with the notion that laboratory contamination has occurred during the extended co-cultivation periods required to recover BDV from samples with low viral titres [153].

Multicentre controlled studies are in progress to assess the epidemiology of human Bornavirus infection. Whether BDV will be demonstrated to be an important human pathogen remains to be determined; nonetheless, this unusual pathogen has already provided intriguing insights into molecular virology and models for investigating mechanisms by which CNS infections and immunity can result in disturbances of brain development and behaviour.

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