Borna Disease Virus and its Hosts

Studies on Virus-Host Interactions and Virus Detection

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Cover: *The colourful BDV-infection*. BDV P *in situ* PLA of a horse with BD, where BDV P is red, glial cells are green, and nuclei are blue.
(photo: Karl-Johan Leuchowius)

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Abstract

Borna disease virus (BDV) is an RNA-virus persistently infecting the central nervous system of, and thereby causing neurological disorders in, several animal species, possibly including humans. Although several aspects of BDV and its pathologies have been studied for more than 100 years, further elucidation of the epidemiology and pathogenesis, and the development of reliable detection methods, is needed.

This thesis describes the relationship between BDV and its hosts, with special emphasis on virus-host interactions and virus detection. The aims were to improve current detection methods, and to increase the knowledge of virus-host interactions, especially in naturally infected animals. First, a real-time RT-PCR assay for BDV detection was developed; and second this method, as well as serology, was evaluated for use when diagnosing Borna disease (BD) in cats. These studies suggest that serology is the most useful tool to further strengthen a clinical suspicion of feline BD, whereas molecular diagnostics, using blood samples, can be added for cats showing mild neurological signs.

In the third study, phosphorylated BDV P and previously reported BDV-host protein-protein interactions were detected in cell cultures, as well as in experimentally and naturally infected animals, by the use of an *in situ* proximity ligation assay. These protein-protein interactions most likely interfere with signaling pathways of the host, enabling BDV to establish a persistent infection.

The type II interferon (IFN-γ) is an important key factor of the antiviral host immune response, involved in non-cytolytic clearance of BDV. In the final study, IFN-γ mRNA expression was studied in feline BD. A high expression was seen in these cats, as compared to non-infected controls. This expression was higher in cats showing mild neurological signs, and in these cats also moderate-severe pathological lesions were found. These data point to more direct effects of BDV in disease development than has previously been considered.

In conclusion, this thesis provides an overview of the current knowledge of BDV, and contributes novel data on virus-host interactions and virus detection.

Keywords: Borna disease virus, staggering disease, persistent infection, protein-protein interactions, IFN-γ, *in situ* proximity ligation assay, diagnostics

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Dedication

To my family

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Wensman, J.J., Thorén, P., Hakhverdyan, M., Belák, S., and Berg, M. (2007). Development of a real-time RT-PCR assay for improved detection of Borna disease virus. *Journal of Virological Methods* 143(1), 1-10.

II Wensman, J.J., Jäderlund, K.H., Gustavsson, M.H., Hansson-Hamlin, H., Karlstam, E., Lilliehöök, I., Öström, I-L.Ö., Belák, S., Berg, M., and Holst, B.S. (2011). Diagnostic markers in feline Borna disease. Submitted.

III ⋆Wensman, J.J., ⋆Leuchowius, K-J., Yan, J., Berg, A-L., Ludwig, H., Bode, L., Belák, S., Landegren, U., Söderberg, O., and Berg, M. (2011). Visualization of Borna disease virus protein interactions with host proteins using *in situ* proximity ligation assay. (Manuscript).

IV Wensman, J.J., Ilbäck, C., Hjertström, E., Blomström, A-L., Gustavsson, M.H., Jäderlund, K.H., Ström-Holst, B., Belák, S., Berg, A-L., and Berg, M. (2011). Expression of interferon gamma in brain tissues of cats with natural Borna disease virus infection. *Veterinary Immunology and Immunopathology* 141(1-2), 162-167.

⋆ Equal contribution.

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Abbreviations

ABV	Avian Bornavirus
ANS	Autonomic nervous system
BD	Borna disease
BDV	Borna disease virus
cDNA	Complementary DNA
CNS	Central nervous system
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
G/GP/gp	Glycoprotein
GABA	γ-aminobutyric acid
GABA-R	GABA-receptor
GABARAP	GABA-receptor associated protein
IFA	Immunofluorescence assay
IFN	Interferon
IHC	Immunohistochemistry
ISH	*In situ* hybridization
kDa	kilo Dalton
L	Large protein, viral RNA-dependent RNA-polymerase
M	Matrix protein
N	Nucleoprotein
NES	Nuclear exportation signal
NLS	Nuclear localization signal
ORF	Open reading frame
P	Phosphoprotein
PCR	Polymerase chain reaction
PLA	Proximity ligation assay
PDD	Proventricular dilatation disease
RNA	Ribonucleic acid

RNP	Ribonucleoprotein or nucleocapsid
rRT-PCR	Real-time RT-PCR
RT-PCR	Reverse transcriptase – PCR
X	p10 protein, non-structural protein of BDV

Introduction

In the 18th century, a neurological disease in horses, at that time called "Kopfkrankheit der Pferde" (head disease of horses), was described in a German textbook (reviewed in the article of Dürrwald & Ludwig, 1997). This disease had been known for a long time in Germany, especially in the southern and southeastern parts, where it occasionally occurred (Zwick, 1939). After severe losses around the city of Borna (Saxony) in the 1890's, the disease was thereafter known as "Bornasche Krankheit" (Borna disease, BD). In 1907, the Ministry of Home Affairs of the Kingdom of Saxony decided that the disease should be thoroughly investigated, with the aim to characterize its clinical signs, pathology and etiology. The clinical signs and pathology were then well characterized by Schmidt in 1912, and by Joest and Degen in 1911. Several attempts to establish the etiology behind BD were made. At first, bacterial causes of the disease were proposed; however, based on the lack of presence of a purulent inflammation, the proposed cause was changed to a bacterial toxin (reviewed by Zwick, 1939).

At the end of the 19th century, the first viruses were discovered: Ivanovsky and Beijerinck described the tobacco mosaic virus, and Loeffler and Frosch the first animal virus (foot-and-mouth disease virus) (Murphy *et al.*, 1999). The first successful attempts to transmit BD from a diseased horse to rabbits were made by Zwick and Seifried in 1924-25 (Zwick & Seifried, 1925), and a few years later Zwick and his co-workers had convincing evidence for a viral etiology of BD.

In the early history of BD research, studies of the pathogenesis, viral entry and secretion, as well as epidemiology, clinical signs and treatment, were in focus. This focus of the BD research has not changed dramatically over the last century, but still many questions within these fields have to be answered. This thesis contributes with improved detection methods and novel information about BDV-host interactions.

Borna Disease Virus

Borna disease virus (BDV) was first considered to be the etiological cause of BD in 1928. Through successful transfer of filtered brain suspensions from a horse with BD to rabbits, and thereafter by several passages in rabbits (Zwick *et al.*, 1928; Zwick & Seifried, 1925), it was concluded that a virus was the causative agent of BD. However, at that time the characteristics of viruses and the structures of viral particles (virions) were unknown.

The characterization of BDV was begun by Zwick and others, especially regarding physical properties, and how the virus could be inactivated (Zwick, 1939; Nicolau & Galloway, 1928; Zwick *et al.*, 1928). The size was established to be around 85-125 μm, values that are still valid today (de la Torre, 2002; Zwick, 1939). In the late 1960's, viral antigens could be visualized by immunofluorescence (Wagner *et al.*, 1968), and a few years later successful cultivation of BDV in tissue culture was performed (Ludwig *et al.*, 1973; Mayr & Danner, 1972). It was suggested that BDV was an RNA-virus (Danner, 1977; Ludwig & Becht, 1977), mainly associated with the infected cells and only to a minor extent released from the cells (Ludwig & Becht, 1977). The first electron micrographs showed spherical particles (Ludwig & Becht, 1977), which was later confirmed (Kohno *et al.*, 1999; Zimmermann *et al.*, 1994a). For a long time, the sequence and organization of the virus genome was unknown. It was not until the 1990's that the first full-genome sequences, and the organization of the BDV genome, were established (Briese *et al.*, 1994; Cubitt *et al.*, 1994a).

Genome Organization

BDV is a non-segmented, non-enveloped virus with a genome of single-stranded negative sense RNA of around 8900 nucleotides in length (Lipkin & Briese, 2006; Briese *et al.*, 1994; Cubitt *et al.*, 1994a). On the basis of its unique nuclear site of replication, compared to other animal viruses within the order of *Mononegavirales* (Briese *et al.*, 1992), BDV is the sole member of the *Bornaviridae* family. The genome is organized in a similar manner to other members of the order of *Mononegavirales*, i.e. N, P, M, G and L (Figure 1). In addition, like most members of the *Paramyxoviridae* family, BDV has a small non-structural gene, designated X, which is located as an over-lapping open reading frame (ORF) together with the P gene (Pringle, 2005; Jordan & Lipkin, 2001).

BDV has three transcription units that encode six ORFs (Jordan & Lipkin, 2001), and exploits the cellular splicing mechanisms to efficiently use its comparatively short genome (Figure 1) (Cubitt *et al.*, 1994b; Schneider *et al.*, 1994). The first ORF in the first transcription unit results in the nucleoprotein (N), whereas the second transcription unit contains two overlapping ORFs for the phosphoprotein (P), and the p10 or X protein (Tomonaga *et al.*, 2002; Jordan & Lipkin, 2001). The third transcription unit is spliced differently, and also has different transcription initiation and termination signals, enabling polymerase read-through during transcription, which results in expression of the matrix protein (M), the glycoprotein (G), and the large protein or RNA-dependent RNA-polymerase (L) (Tomonaga *et al.*, 2002; Jordan & Lipkin, 2001).

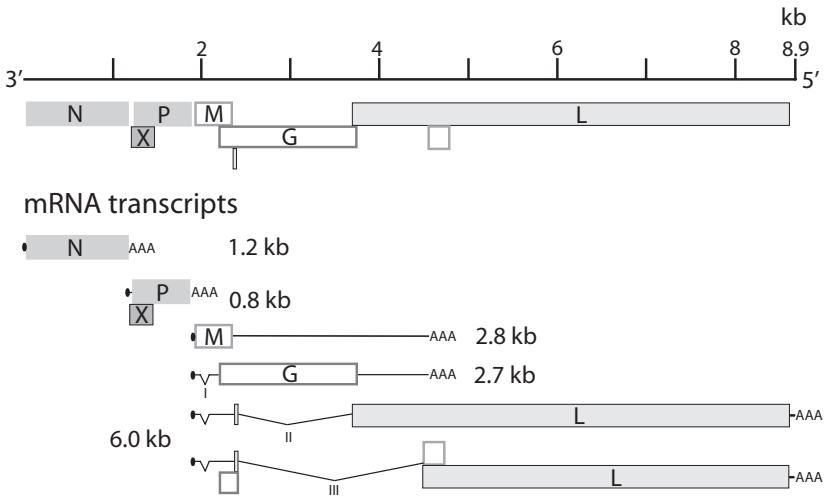

Figure 1. Map of genome organization and protein-coding mRNA transcripts of BDV. The BDV genome is comparatively short, and therefore BDV uses alternative transcription strategies, like over-lapping ORFs and usage of host cellular splicing mechanisms. Modified from Tomonaga et al., *(2002).*

The genome of BDV is highly conserved, and so far two genotypes have been observed with approximately 15% differences at the nucleotide level (Nowotny *et al.*, 2000). Some observations indicate that BDV isolates cluster into separate geographical regions based on their genetic composition (Kolodziejek *et al.*, 2005), though other studies have not confirmed this (Bode, 2008; Wensman *et al.*, 2008). The conserved genome has led to the conclusion that BDV is an evolutionarily old virus, which has further been

supported by recent findings of BDV-like elements in the genome of different mammals including the human genome (Belyi *et al.*, 2010; Horie *et al.*, 2010).

Recently, a more divergent Bornavirus with similar genome organization has been detected in psittacine birds, designated Avian Bornavirus (ABV) (Honkavuori *et al.*, 2008; Kistler *et al.*, 2008).

Viral Proteins

The six polypeptides encoded in the BDV genome each have important functions in the viral life cycle.

Nucleoprotein

The nucleoprotein (N) is the most abundant viral protein, and is mainly located inside the nucleus (de la Torre, 2002). Besides the genomic RNA, N is the main component of the nucleocapsid or ribonucleoprotein (RNP) complex. BDV N and the BDV-RNA form polymers (Hock *et al.*, 2010), together forming the backbone of the RNP.

BDV N also interacts with P (Berg *et al.*, 1998b), and has an important role, together with P, in the intracellular transport of the RNPs to and from the nucleus (Kobayashi *et al.*, 2001).

Phosphoprotein

In other negative-stranded non-segmented RNA-viruses, the phosphoprotein (P) is an important co-factor to the polymerase complex in the processes of transcription and replication. However, unlike these other viral phosphoproteins, BDV P down-regulates the activity of the viral polymerase upon phosphorylation (Schmid *et al.*, 2007). The phosphorylation of P is still needed for efficient viral spread in infected cells (Schmid *et al.*, 2010), indicating important functions of P in viral transmission.

BDV P is phosphorylated at serine residues mainly by protein kinase Cε (PKCε), but to a lesser extent also by casein kinase II (CKII) (Schwemmle *et al.*, 1997). In infected cells, P forms homomers, either as tri- or tetramers (Hock *et al.*, 2010; Schneider *et al.*, 2004). The phosphorylated P multimer can interact with L (Walker *et al.*, 2000); however, N not bound to the RNP complex can block this interaction (Schneider, 2005).

BDV P can also interact with X (Schwemmle *et al.*, 1998), and does so preferably as a monomer, which indicates that X plays a role in multimerization of P (Schneider, 2005).

Matrix Protein

The matrix protein (M) of BDV composes a shell or an outer layer of the RNP, likely protecting the genomic RNA and the other nucleocapsid proteins. BDV M forms tetra- or octamers (Kraus *et al.*, 2005; Stoyloff *et al.*, 1997), which are a part of the RNP complex by interaction with P without inhibitory effects of the polymerase activity (Chase *et al.*, 2007). Moreover, M binds to single-stranded RNA and interacts with lipid membranes (Neumann *et al.*, 2009), suggesting a key role in assembly of RNPs and viral particles similar to other negative-stranded RNA-viruses.

Antibodies towards M neutralize BDV infectivity (Stoyloff *et al.*, 1998; Hatalski *et al.*, 1995), indicating that M is present on the surface of the infectious virus particle. However, BDV also spreads as RNPs, from cell to cell inside the CNS as well as in cell culture (Clemente & de la Torre, 2007; de la Torre, 2002; Gosztonyi *et al.*, 1993). Hence, the neutralizing effect of anti-M antibodies most likely is due to neutralization of infectious RNPs.

Glycoprotein

Glycoproteins (G) of viruses are membrane proteins important for viral attachment to cellular receptors and viral entry into the host cell.

In BDV, G is a glycosylated protein with a molecular weight of about 94 kDa (gp94) (Schneider *et al.*, 1997), which is a precursor molecule needed to be cleaved by the cellular protease furin into two biologically active proteins, GP-1 and GP-2 (Richt *et al.*, 1998). GP-1 is responsible for attachment to the host cell surface by binding of (a) yet unidentified cellular receptor(s) (Clemente *et al.*, 2010; Perez *et al.*, 2001). However, one potential player is BiP (immunoglobulin heavy chain-binding protein), which is an endoplasmic chaperone also expressed on the cell surface (Honda *et al.*, 2009). Upon receptor binding, BDV is taken up by the host cell through endocytosis (Clemente & de la Torre, 2009; Perez *et al.*, 2001). In the early endosome inside the cytoplasm, GP-2 mediates the pH-dependent fusion of the viral and endosomal membranes to release the RNP (Clemente & de la Torre, 2009; Gonzalez-Dunia *et al.*, 1998). Antibodies against BDV G have neutralizing activity (Gonzalez-Dunia *et al.*, 1997; Schneider *et al.*, 1997).

Large Protein or RNA-Dependent RNA-Polymerase

The large (L) protein of BDV is an RNA-dependent RNA-polymerase of around 190 kDa (Walker *et al.*, 2000). L has the possibility to translocate into the nucleus by itself (Walker & Lipkin, 2002; Walker *et al.*, 2000), though other viral proteins within the RNP further facilitate this nuclear

translocation (de la Torre, 2002). Cellular kinases phosphorylate L, which probably is a part of the regulation of the polymerase activity (Walker *et al.*, 2000).

For successful transcription and replication, several BDV proteins (N, P, and L) are needed to form a polymerase complex (Schneider, 2005). The model for how this complex works, is most likely the same as that proposed for other non-segmented negative-stranded RNA-viruses, like Sendai virus (Hock *et al.*, 2010; Schneider, 2005; Curran, 1998). Phosphorylated BDV P negatively regulates the activity of the polymerase complex (Schmid *et al.*, 2007), which could be contributed by the binding of X to P (see below; Poenisch *et al.,* 2004).

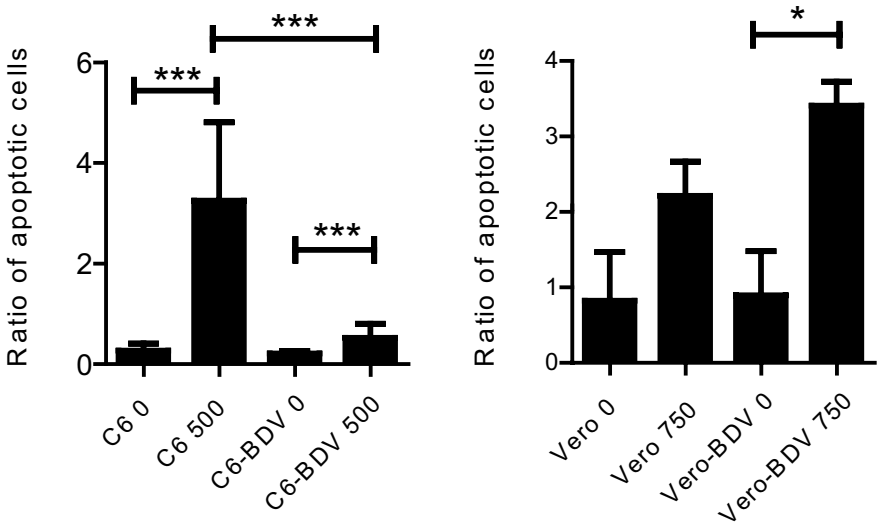

Figure 2. Apoptosis resistance in BDV-infected C6 cells, but not in BDV-infected Vero cells after H_2O_2 treatment. Cells were either mock-treated (0) or treated with 500 (C6) or 750 (Vero) μM of H_2O_2 for 48 h, harvested and incubated with Annexin V antibody (apoptotic cell marker) and propidium iodide (necrotic cell marker). Subsequently, fluorescent activated cell sorting (FACS) was performed, and the ratio of apoptotic cells (R) then calculated according to the equation R=([necrotic+apoptotic cells]+apoptotic cells)/living cells. The asterisks indicate statistically significant differences (two-tailed t-test), where ⋆ is $p<0.05$, and ⋆⋆⋆ $p<0.001$.

X Protein

Like other members of the order of *Mononegavirales* (Pringle, 2005), BDV also expresses a small non-structural or accessory protein (Schwardt *et al.*, 2005). This protein is called X or p10, based on its molecular weight of 10

kDa, and co-localizes in the nucleus together with N and P (Wehner *et al.*, 1997), by interaction with P (Wolff *et al.*, 2000; Schwemmle *et al.*, 1998). BDV X seems to be a multifunctional protein. Besides its involvement in the regulation of the polymerase complex activity (Poenisch *et al.*, 2008; Poenisch *et al.*, 2007; Poenisch *et al.*, 2004), X also inhibits apoptosis, thereby promoting a persistent infection (Poenisch *et al.*, 2009). The mechanism for this apoptosis resistance is as yet unknown, though X seems to have to localize to the mitochondrion to exercise this resistance (Poenisch *et al.*, 2009).

However, there are cell line differences in the X-induced apoptosis resistance of BDV-infected cells. In a rat astrocytoma (C6) cell line, BDV-infected cells are clearly resistant to apoptosis stimuli (Figure 2, left diagram; Poenisch *et al.,* 2009), whereas a green monkey kidney (Vero) cell line shows no BDV-induced apoptosis resistance (Figure 2, right diagram).

BDV-Like Elements in Mammalian Genomes

Some viruses are known to be able to insert parts of their genomes into the genomic DNA of the host. For some viruses, like retroviruses, this strategy is needed for their replication. Other viruses, like herpesviruses and parvoviruses, most likely use this capacity as a means to avoid the host immune response, by establishing a form of latent or persistent infection (Liu *et al.*, 2011; Morissette & Flamand, 2010), but it could also be a way to exchange genetic material. The integration of viral genes into the host genome can lead to different pathological changes, such as tumor formation, and possibly even autoimmunity (Morissette & Flamand, 2010). During the course of evolution, viral hosts likely have gained new genes, beneficial for their survival, through this gene exchange, either by getting novel functional proteins and/or as a way to acquire immunity to these viruses (Koonin, 2010).

Until recently, only retroviruses and DNA-viruses were known to have the ability to incorporate into the genome of the host cell. BDV-like elements have now been found integrated into the genome of different mammals, including humans (Belyi *et al.*, 2010; Horie *et al.*, 2010). These integration events happened millions of years ago (Belyi *et al.*, 2010; Horie *et al.*, 2010), though they can also occur in acute infection of different animals (Kinnunen *et al.,* 2011; Horie *et al.*, 2010). Furthermore, elements from other RNA-viruses within the order of *Mononegavirales*, Ebola and Marburg viruses, have also been found incorporated into the genomes of mammalian hosts (Belyi *et al.*, 2010). Some of these elements result in protein expression (Belyi *et al.*, 2010; Horie *et al.*, 2010). These findings

have led to renewed discussions about the relationship between BDV and possible human infections (Kinnunen, 2011; Feschotte, 2010; Tomonaga, 2010).

Diseases Caused by Bornaviruses

Most mammals and birds seem to be susceptible to BDV infection, although not all experimental infections are followed by disease. Infection occurs naturally in most mammals, as well as in birds. In the following sections, the most common natural host species of BDV leading to disease will be described. Experimental infection in rats will also be discussed briefly.

Borna Disease in Horses, Sheep and Cattle

The horse was the first recognized host species of BDV, and it was from brain tissues from horses with BD that Zwick and his co-workers could infer a viral etiology (Zwick, 1939; Zwick *et al.*, 1928; Zwick & Seifried, 1925). Around the same time, BDV was found in sheep and cattle (reviewed in Zwick, 1939; Nicolau & Galloway, 1928). Before the etiology of BD was determined, the clinical signs in horses were carefully characterized (Schmidt, 1912). This thorough investigation of over 400 cases, as well as similar recent studies by others (reviewed in Heinig, 1969; Richt *et al.,* 2000; Ludwig *et al.,* 1985), have established the following clinical signs:

The early signs of BD are disturbances in feed intake, like arrested eating, fever and different degrees of somnolence. Mild colic signs and/or irregular defecation, alternately with constipation and diarrhea, are commonly seen. In stallions and geldings, a continuously prolapsed penis without urination is common (Figure 3). Hypersensitivity and muscular twitches occur in both the head and extremities, as well as gait disturbances and hesitation when jumping over hurdles.

These signs become progressively aggravated over the following days. The sick horse gets more somnolent, with abnormally lowered head most often pressed against the wall or supported by the crib, and its eyes closed (Figure 4, to the right). The gait disturbances get more pronounced, and if the horse is allowed to move at will it frequently moves in circles, always in the same direction (Figure 4, to the left). If the legs are manipulated, horses with BD will sometimes keep standing with over-crossed legs without trying to make corrections, indicating postural deficits. Muscular convulsions are common, for example in the chewing muscles, causing bruxism and problems with feed intake. Involuntary eye movements

(nystagmus), different sized pupils and blindness are also common signs (Richt *et al.*, 2000; Heinig, 1969; Schmidt, 1912).

Figure 3. Horse with clinical signs of BD. The horse shows apathy, weight loss, and a continuously prolapsed penis, all typical signs of BD. Received from Hanns Ludwig, Berlin, Germany.

In the end-stage, muscles of the head and extremities get paralyzed. Paralysis is usually the cause of death, by hindering feed and water intake (paralysis of the tongue, chewing, and/or swallowing muscles), and/or by immobilizing the animal. Fever is also seen frequently in the end-stage of the disease (Schmidt, 1912). More atypical and milder clinical signs, like recurrent colic, gait disturbances and behavioral changes, connected to BDV-infection have also been reported (Berg *et al.*, 1999a; Bode *et al.*, 1994a). The duration of disease is mostly 1-3 weeks, but longer durations can be seen (Heinig, 1969). In some cases, complete or partial recovery occurs spontaneously, sometimes followed by relapses and death (Heinig, 1969; Schmidt, 1912). The prognosis of BD is usually considered to be bad, and a mortality rate of 75-95% has been described (Heinig, 1969).

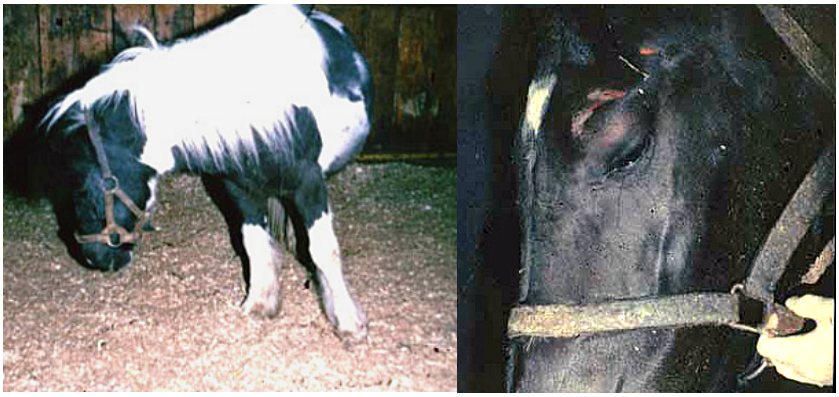

Figure 4. Horses showing typical clinical signs of BD. *Left:* A pony showing circular movement. Ponies and haflingers seem to be more susceptible to BDV-infection, and show more severe signs with faster progression (Liv Bode and Hanns Ludwig, personal communication). *Right:* A horse with BD in the end-stage of the disease. This horse is somnolent with lowered head, and head injuries after throwing it against the wall. This horse was positive for BDV by FACS analysis (Bode *et al.*, 1994c). Received from Hanns Ludwig, Berlin, Germany.

However, reports where novel diagnostic methods have been used for detection of antibodies and/or antigen have questioned the high mortality rate of BD, since many horses seem to have sub-clinical or atypical BDV-infection (Bode, 2008; Dieckhöfer, 2008; Ludwig & Bode, 2000; Berg *et al.*, 1999a).

In sheep, a similar clinical picture is seen as for horses, although up to 20% of a herd can develop the disease, while only sporadic cases occur in horse stables (Ludwig & Kao, 1988). Early signs in sheep are social behavioral changes and apathy (Heinig, 1969). Hyperesthesia in the lumbosacral region is also common. As the disease progresses, decreased feed intake, bruxism and circular movement are seen (Figure 5). The incubation time is several weeks, and the duration of disease is around 4-10 days, with around 90% mortality (Ludwig & Kao, 1988; Heinig, 1969).

Besides the initial reports of BD in cattle (Zwick, 1939; Nicolau & Galloway, 1928), demonstrated by transmission of the disease to laboratory animals, there seems to be only sporadic occurrence in this species. Hence, BDV-infection of cattle has been considered as a possible event (Mayr & Danner, 1978). In more recent cases, similar clinical signs as for horses and sheep have been reported, such as decreased feed intake, gait disturbances including circular movement, and finally in some cases paresis or paralysis (Okamoto *et al.*, 2002a; Bode *et al.*, 1994b; Caplazi *et al.*, 1994).

BD has also been observed in other ungulates (donkeys, mules and hinnies), as well as in goats and rabbits, with similar signs as for horses and sheep (Caplazi *et al.*, 1999; Metzler *et al.*, 1978; Heinig, 1969).

Figure 5. Sheep with BD in the end-stage of the disease. This sheep is severely ataxic and paretic. The same sheep is shown from another angle in Figure 5 of Ludwig & Bode (2000). Received from Hanns Ludwig, Berlin, Germany.

Bornavirus Infection in Birds

Recently, two research groups in the United States independently found a BDV-like virus in psittacine birds suffering from proventricular dilatation disorder (PDD), designated Avian Bornavirus (ABV) (Honkavuori *et al.*, 2008; Kistler *et al.*, 2008). The genome organization of ABV is similar to that of BDV. Recently, successful experimental infection with an ABV isolate of two different bird species has been reported, thereby fulfilling Koch's postulates (Payne *et al.*, 2011; Gray *et al.*, 2010).

PDD is a devastating disease in wild and captive exotic birds, previously known as macaw wasting disease, where the birds show gastrointestinal (GI) and/or neurological signs (Gregory *et al.*, 1994). The pathology is mainly characterized as a lymphocytic inflammation of the ganglia of the GI tract and/or CNS. The most common clinical signs are depression, weight loss, passage of undigested feed in the feces, gait disturbances, seizures, and decreased or absent postural reactions.

Not all birds positive for virus and/or antibodies develop disease, but instead can be healthy carriers and transmitters of ABV (Hoppes *et al.*, 2010). The routes of transmission are likely to be fecal-oral, but air-borne transmission could also occur. ABV-RNA has been found in nasal, choanal, and cloacal swabs, as well as in feces and in feathers. However, only feces has been confirmed to contain infectious virus, because mallards that had eaten fecal droppings from infected cockatiels two weeks later shed viral RNA in their feces (Hoppes *et al.*, 2010).

BDV-Infection in Wildlife

A few wildlife species have been shown to be susceptible to BDV-infection followed by clinical signs, and even more species have been shown to carry virus (viral RNA and/or antigen), or BDV-specific antibodies without obvious clinical signs. The latter group will be discussed more in detail below, in their role as potential BDV reservoirs.

The first note of BD or a BD-like disease in wildlife was reported from Germany in the early 20th century (Zwick, 1939; Nicolau & Galloway, 1928). A deer was showing strange behavior when a hunter was approaching, and was subsequently shot. At necropsy, the typical histological lesions of BD were found, including the presence of intra-nuclear Joest-Degen inclusion bodies.

In Sweden, a free-ranging lynx was shot because of its abnormal behavior (Degiorgis *et al.*, 2000). At necropsy, a non-purulent inflammation of the CNS was found. Neurons and glial cells were positive for BDV-RNA and BDV-antigen, using *in situ* hybridization (ISH) and immunohistochemistry (IHC). Furthermore, BDV-RNA was found by RT-PCR, and partial sequence analysis revealed a close genetic relationship to other Swedish BDV strains (96.2-97.7% amino acid identity), as well as to laboratory strains (98.5% amino acid identity), yet differences were present (Wensman *et al.*, 2008; Degiorgis *et al.*, 2000).

Experimental BDV-Infection in Rats

Depending on the age and immune-competence of the rat, as well as the passage number of the virus, the outcome of BDV-infection is highly variable (Gosztonyi & Ludwig, 1995). In newborn rats, BDV-infection leads either to persistent infection with mild behavioral changes, with or without an inflammatory reaction, or to progressive neurological signs with fatal outcome in the absence of inflammation, depending on the passage number and the species used for virus adaptation. In weanlings and adult rats, acute encephalitis with the classical neurological signs of BD is seen, and the animals die within 1-4 months. However, around 5-10% of these rats survive the acute disease, and develop obesity and aggressive behavior (Ludwig, 2008; Carbone *et al.*, 1987; Ludwig *et al.*, 1985; Hirano *et al.*, 1983). Upon intranasal infection, weanlings and adult rats develop an acute or sub-acute disease, with a severe inflammatory infiltration. In natural infection and upon experimental infection of adult rats, BDV shows no or just a few signs of cytopathogenicity; most neurons are intact despite a heavy inflammation. However, in persistent infection BDV causes severe pathological alterations of certain parts of the brain, thereby showing

cytopathic effects. Thus, BDV has dual capacities: to induce cell death and neuronal degeneration, and persistence without obvious damage to the host cells.

Borna Disease in Cats and Dogs

In the 1970's, a fatal neurological disorder in cats was reported from certain parts of Sweden (Kronevi *et al.*, 1974). The clinical signs were characterized by gait disturbances (Figure 6), such as ataxia and staggering movement, and by behavioral changes; thereby the disease got known as staggering disease (Sw. *vingelsjuka*). A viral etiology was suspected, because of the non-purulent inflammation of the CNS, and thus thorough efforts to isolate a virus were made. However, the etiology remained unknown until the 1990's, when antibodies towards BDV were found in diseased cats (Lundgren & Ludwig, 1993). This finding pointed towards BDV-infection, which was further supported by the pathological lesions found in the same regions of the CNS as previously found in, for example, horses with BD (Lundgren, 1992; Gosztonyi & Ludwig, 1984).

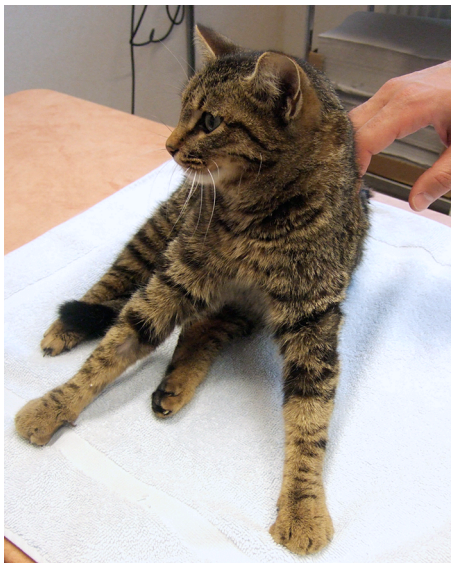

Figure 6. Cat with staggering disease or feline BD. The cat was severely ataxic, and without support it fell over on its side. This cat is included in Paper II and IV. Photo: Jonas J Wensman.

Moreover, clinical signs are strikingly similar to BD in horses and sheep: initially cats have fever, apathy and reduced appetite, followed by staggering and circling movements, behavioral changes, and finally, after a duration of 1-4 weeks, paresis and/or paralysis (Lundgren, 1992; Kronevi *et al.*, 1974). Other minor signs in common with horses, like constipation and impaired

vision, are also seen. However, a clear etiology was not established until a feline BDV was isolated (Lundgren *et al.*, 1995b), and used in experimental infection in cats, inducing similar clinical signs and pathological lesions, thereby fulfilling the postulates of Koch (Lundgren *et al.*, 1997). Since then, BDV-RNA, -antigen, and/or BDV-specific antibodies have been found in cats with staggering disease, further strengthening the etiology (Wensman *et al.*, 2008; Johansson *et al.*, 2002; Berg & Berg, 1998).

Atypical clinical signs have also been reported (Berg & Berg, 1998). In this case, a cat had muscular fasciculation, after a short initial period of reduced appetite and apathy. After a few weeks, the signs progressed and the cat showed decreased postural reactions. Upon histological investigation, no inflammatory reaction was seen inside the CNS, although BDV-RNA was found *in situ* and by RT-PCR.

Cases of full or partial recovery are also seen, mostly followed by relapses (Hultin Jäderlund, 2003; Lundgren, 1995). In a few cases, an obesity syndrome in recovered cats has been observed (Lundgren, 1995), similar to what is seen in experimentally infected rats (Ludwig *et al.*, 1985).

Staggering disease or similar neurological disorders have also been reported in other countries (Kamhieh & Flower, 2006). In Austria, cats with staggering disease were sero-positive for BDV (Nowotny & Weissenböck, 1995). Brain suspensions from those cats were inoculated into rabbits, which sero-converted but did not develop signs of BD. Later, BDV-RNA was also found in an Austrian cat (Berg & Berg, 1998).

BDV-infection has been found in dogs showing neurological signs, but only two cases have been scientifically reported (Okamoto *et al.*, 2002b; Weissenböck *et al.*, 1998). In the Austrian case, the dog was fatigued and had a loss of appetite, followed by severe undefined neurological signs (Weissenböck *et al.*, 1998). In the case from Japan, the dog initially showed hypoesthesia and tremor, and after 10 days presented with circling movement, dilated pupils and salivation (Okamoto *et al.*, 2002b). In both cases, a non-purulent inflammation of the CNS was seen, and BDV-antigen and –RNA were found in cells. Even though these reports show that dogs can be infected by BDV and develop clinical signs, BDV-infection in dogs needs to be further scrutinized.

To my knowledge, at least two dogs have been sero-positive for BDV in Sweden. These dogs were presented with gait disturbances.

Pathogenesis of BDV

Based on the careful characterization of the pathology of BD in horses, Joest and Degen proposed already in the early 20th century that BDV enters the CNS through the olfactory epithelium and olfactory nerve (Joest & Degen, 1911). They also suggested that the virus spreads from neuron to neuron. Some years later, Zwick and his co-workers proposed that BDV is not only taken up by the olfactory epithelium, but also secreted from there (Zwick *et al.*, 1928). They successfully transmitted BDV from suspensions of olfactory epithelium, taken from experimentally infected rabbits at the end-stage of the disease, to naïve rabbits by intra-cerebral injection. In addition, nasal secretions from an experimentally infected horse were transferred intra-nasally to a rabbit. This rabbit developed mild clinical signs and mild pathological lesions, and brain suspension from this rabbit successfully transmitted the disease to other rabbits in two passages. Taken together, this pioneering work in the first decades of the 20th century, clearly showed that BDV most likely enters into neural cells of the olfactory epithelium, is transported via the olfactory nerve, and also is transported back to the olfactory epithelium from the CNS and secreted in the nasal secretions of infected animals.

More recent studies confirm that BDV most likely enters the nervous system through the open nerve-endings in the olfactory epithelium and/or oro-pharyngeal mucosa (Sauder & Staeheli, 2003; Morales *et al.*, 1988). Viral entry through nerves in the gastrointestinal system has also been discussed (Heinig, 1969), based on successful experimental oral infections. Cell entry occurs through the binding of GP1 to a cellular receptor, which guides clathrin-mediated endocytosis (Clemente & de la Torre, 2009; Perez *et al.*, 2001; Gonzalez-Dunia *et al.*, 1998). Thereby, BDV is taken up by the cell through encapsulation into endosomes, and can be released as RNPs from the early endosome by a pH-dependent fusion mediated by GP2 (Clemente & de la Torre, 2009; Gonzalez-Dunia *et al.*, 1998). How the RNPs are transported to the replication site inside the nucleus is not known. Even though the exact cellular receptor, to which BDV GP1 binds, has not yet been identified, several host factors important for viral entry have been recognized (Clemente *et al.*, 2010). Among these are some cell surface proteins, for example a subunit of certain GABA-receptors. Whether these *in vitro* findings reflect the situation in infected animals needs further elucidation.

BDV probably uses the axonal transport system of macromolecules for transport to the CNS, and reaches the olfactory bulb around 4-6 days after experimental intra-nasal infection (Gosztonyi, 2008; Gosztonyi & Ludwig,

1995; Carbone *et al.*, 1987). Thereafter, BDV antigen can be detected along the higher olfactory pathways within the limbic system, later disseminating to the entire cortical area (Gosztonyi, 2008). Inside the CNS, the viral spread is trans-neural, most likely through RNPs, and not as whole virus particles (Clemente & de la Torre, 2007; Gosztonyi *et al.*, 1993). The glutamate kainate 1 (KA-1) receptor has been proposed as the BDV receptor in CNS (Gosztonyi, 2008; Gosztonyi & Ludwig, 2001). Clinical disease appears when viral antigens are expressed in the neurons of the hippocampus, along with an inflammatory reaction (Carbone *et al.*, 1987). Therefore, the incubation time depends on the route of infection. In an experimental intra-nasal infection of rats, the incubation time was around 20 days (Carbone *et al.*, 1987), whereas it in horses can take up to six months (Mayr & Danner, 1974). The infectious dose likely contributes to the incubation time.

One to two months after experimental intra-nasal infection, BDV starts to spread centrifugally to the spinal cord, and to the cranial and peripheral nerves, including nerves of the autonomic nervous system (ANS) (Gosztonyi, 2008; Gosztonyi & Ludwig, 1995). BDV also spreads to retinal neurons, causing neuronal degeneration, which leads to blindness (Dietzel *et al.*, 2007). From the nerves of ANS, more or less every visceral organ gets infected after another one or two months. There, BDV actively replicates, which results in secretion of infectious virus particles (Gosztonyi, 2008; Gosztonyi & Ludwig, 1995). For example, infectious BDV has been detected in lacrimal and nasal secretions of naturally infected horses (Richt *et al.*, 2000), urine of experimentally infected rabbits (Zwick, 1939) and rats (Gosztonyi, 2008; Sauder & Staeheli, 2003), in saliva of experimentally infected horses and rabbits (Zwick, 1939), and in milk of experimentally infected rabbits (Zwick, 1939).

Virus-Host Interactions

Immune Responses of the Host

When a virus infects a host cell, a cascade of different actions starts in order to minimize the effects of the intruder. Among these first actions is the shutdown of DNA and RNA synthesis, as well as translation of proteins, thereby obstructing virus replication and production of viral proteins, which the virus uses to interfere with different signaling pathways in the host (see below). The host cell also produces different cytokines (signal molecules), which bind to receptors of neighboring cells (paracrine) and also to the same

cell (autocrine), to induce an antiviral state, thus reducing the possibility for viral infection to spread (Garcia-Sastre & Biron, 2006).

Upon infection, the type I interferons, IFN-α and IFN-β, are important key signal molecules in this first line of defense (the innate immune response), where IFN-β is the most important in the CNS, because of the neurotoxicity of IFN-α (Griffin, 2003). Type I IFNs induce the expression of hundreds of genes, resulting in different host defense mechanisms to infection. Some of these actions result in death of infected cells (cytolysis), to reduce and control an infection (Chawla-Sarkar *et al.*, 2003). The CNS is sensitive to cytolytic virus clearance, as many neurons are non-renewable and essential for the organism. Hence, non-cytolytic ways of clearing a viral infection are important within the CNS (Griffin, 2003; Chesler & Reiss, 2002). Neurons seem to have specific defense mechanisms, driving a viral infection to be non-cytopathic, and stimulating the host immune response to follow non-cytolytic clearance (Patterson *et al.*, 2002). For example, host neurons reduce or block the budding of viral particles, which can result in cytolysis, and viruses have evolved trans-synaptic, non-cytolytic spread of RNPs to evade this response of the host.

The second line of defense (the adaptive immune response) is composed of two main pathways: the humoral immunity, characterized by antibody production, and cell-mediated immunity, consisting of T cells. The cytokines produced initially by the innate immune response attract cells of the adaptive immunity, such as natural killer (NK) cells, CD4+ and CD8+ T cells, and monocytes/macrophages, first to the perivascular tissues, and then also to the brain parenchyma (Griffin, 2003). These immune cells are important for the non-cytolytic clearance of viruses, although different mechanisms are of varying importance when clearing viruses from different cells of the CNS. In neurons, virus clearance is mainly carried out by antibodies, locally produced by B cells, and IFN-γ, produced by T cells and neurons. T cells in combination with IFN-γ are responsible for viral clearance of glial cells. Even though virus clearance by these mechanisms can be effective, viruses can still persist.

A heavy inflammation with cytokine expression affects the normal functions of the CNS adversely, and can produce different neurological signs, including behavioral changes (Capuron & Miller, 2011). Natural BDV infection, and experimental infection of immune-competent animals, leads to the induction of a T cell immune response (Stitz *et al.*, 1995). Thereby, BD is considered to be an immune-mediated disease, though increasing evidence points towards direct virus-induced clinical signs as well (see below).

In perivascular cuffs of experimentally infected rats, CD4+ T helper cells are the most common cell type, whereas cytotoxic CD8+ T cells are more common in the brain parenchyma (Stitz *et al.*, 2002; Hatalski *et al.*, 1998; Deschl *et al.*, 1990). Antibodies, locally produced by plasma cells, can be detected (Deschl *et al.*, 1990), and are probably involved in virus clearance. IFN-γ mRNA is expressed, especially in acute infection (Hatalski *et al.*, 1998) but also in the chronic phase (Shankar *et al.*, 1992), and is crucial for CD8+ T cell-mediated clearance of BDV in experimentally infected mice (Hausmann *et al.*, 2005).

A similar picture is seen in naturally infected horses and cats, which are the only natural hosts of BDV where the immunological responses have been studied so far. Both in horses and cats, CD4+ T cells dominate the perivascular cuffs, whereas CD8+ T cells are more common in the parenchymal tissues, at least in horses (Bilzer *et al.*, 1995; Lundgren *et al.*, 1995a). Overall, CD8+ T cells are less abundant than CD4+ T cells in the brain of BDV-infected cats (Berg *et al.*, 1999b). BDV infection in cats causes an increase in the peripheral CD8+ T cells. This cell population can be divided into two subpopulations, CD8+low and CD8+high, based on the expression of the β-chain, and the CD8+low T cells dominate in the brain (Berg *et al.*, 1999b). The exact difference between these two subpopulations is not entirely known, but it is thought that CD8+low T cells have similar functions to NK cells (Shimojima *et al.*, 2004; Berg *et al.*, 1999b). Plasma cells are found in the brain parenchyma, next to infected neurons in cats (Lundgren *et al.*, 1995a), probably facilitating virus clearance.

Immune Evasion Mechanisms of BDV

To be able to establish a persistent infection, BDV needs to circumvent the host immune response. Several viruses have evolved type I IFN inhibiting properties, since these cytokines are key players in the innate host immune defense (Garcia-Sastre & Biron, 2006). BDV has also developed several ways to inhibit the expression of type I IFNs.

When BDV replicates, the triphosphate group is replaced by a monophosphate at the 5'-end of the genomic RNA (Schneider *et al.*, 2005). Thereby, BDV, as well as ABV, avoids recognition by retinoid inducible gene I (RIG-I), which is an important cytosolic viral sensor and inducer of type I IFN gene expression (Reuter *et al.*, 2010; Habjan *et al.*, 2008). These findings were observed when genomic RNA was transfected into cells. However, it is not known to what extent genomic RNA within the RNP is exposed to RIG-I in natural infections. BDV enters the cell by receptor-mediated endocytosis as an intact viral particle, and is released as RNPs from

the early endosome into the cytoplasm, followed by transport to the replication site inside the nucleus (Clemente & de la Torre, 2009; Gonzalez-Dunia *et al.*, 1998). Between cells inside the CNS and in cell culture, BDV spreads as RNPs (Clemente & de la Torre, 2007; Gosztonyi *et al.*, 1993). Hence, most likely other cellular receptors sensing viral components are important for the recognition of BDV, such as Toll-like receptors (TLRs) 3 or 7/8 inside the endosomes, which recognize double-stranded and single-stranded RNA respectively, or yet unknown viral sensors (Baum & Garcia-Sastre, 2010).

Thus, to evade the host immune response and establish a persistent infection, BDV has evolved other IFN-inhibiting strategies as well. BDV P interferes with the IFN-β mRNA expression by acting as a decoy substrate for phosphorylation by TBK-1 (Unterstab *et al.*, 2005), a cellular kinase activating transcription factors that enhance type I IFN expression.

BDV X also inhibits the type I IFN system, by an as yet unknown mechanism (Figure 7). Furthermore, there seems to be strain variation in the efficiency of inhibition. Other members of *Mononegavirales* have non-structural proteins like X, with similar IFN-inhibiting properties, for example VP35 of Ebola virus (Basler & Amarasinghe, 2009) and V protein of paramyxoviruses (Goodbourn & Randall, 2009).

One cellular defense mechanism upon type I IFN signal transduction is apoptosis induction (Chawla-Sarkar *et al.*, 2003). Therefore, it is important to circumvent apoptosis to establish persistent infection. Certain persistently BDV-infected cell lines are resistant to apoptosis (Figure 2), and it is the X protein that is responsible for this resistance (Poenisch *et al.*, 2009). The exact mechanisms by which X interferes with apoptotic pathways are not known.

BDV has also developed mechanisms to evade the effects of IFN-γ, a key player within the adaptive immune response to viral infections, facilitating viral clearance by non-cytolytic mechanisms (Chesler & Reiss, 2002). One such antiviral effect of IFN-γ is induction of inducible nitric oxide synthase (iNOS), which results in the production of free oxygen radicals, harmful to viruses. In rat astrocytes, BDV P inhibits the expression of iNOS (Peng *et al.*, 2007), thereby overcoming this antiviral host response.

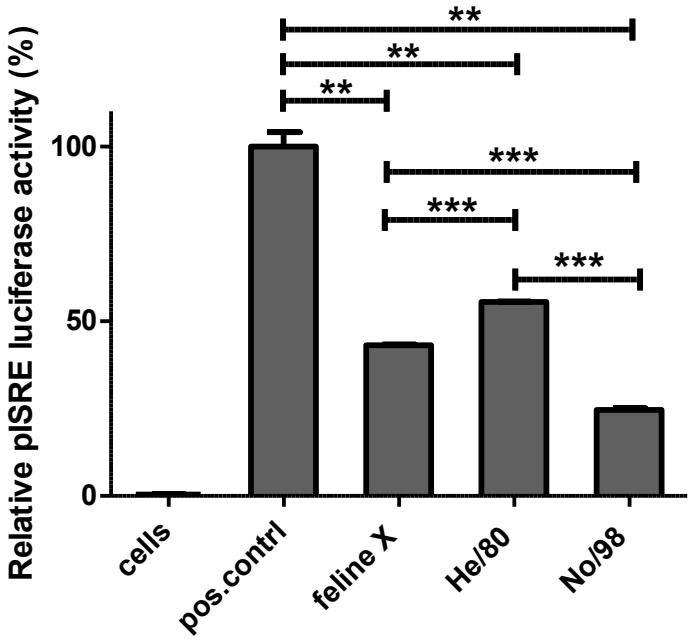

Figure 7. BDV X induced type I IFN inhibition in A549 cells. Cells were co-transfected with an ISRE reporter plasmid and respective BDV X in a flag-tagged vector (1 μg). Twenty-four h post-transfection, type I IFN expression was induced by Sendai virus for 24 h, followed by luminescence reading of the luciferase activity. The luciferase activity was normalized to the positive control, consisting of empty flag-tagged vector. The asterisks indicate statistically significant differences, where ** is $p<0.01$, and *** $p<0.001$.

Protein-Protein Interactions

The proteins of BDV interact with each other, and with the genomic RNA, to form the RNP and the virion, as previously discussed. BDV N interacts with P (Berg *et al.*, 1998b; Schwemmle *et al.*, 1998), which in turn interacts with L and X (Schneider, 2005; Schwemmle *et al.*, 1998), as well as with M (Chase *et al.*, 2007). Besides these interactions, BDV proteins interact with several host cellular proteins (Planz *et al.*, 2009), with potential to interfere with important cellular signaling cascades in favor of viral persistence (Figure 8).

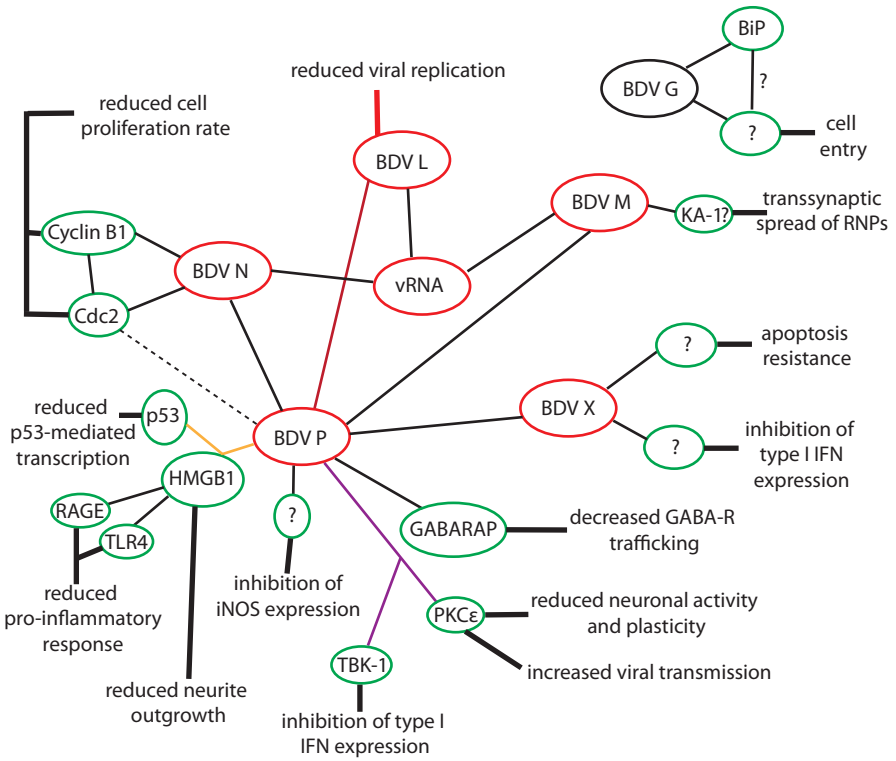

Figure 8. Map of interactions within BDV, between BDV and host cellular proteins, and the biological impact of these interactions. BDV proteins, and viral RNA (vRNA) are marked in red, whereas cellular proteins are in green. Phosphorylated BDV P (i) down-regulates the activity of the viral polymerase (BDV L; connector marked in red), (ii) acts as decoy substrate for TBK-1 and PKCε phosphorylation (purple connector), and (iii) competes with p53 for the same binding site of HMGB1 (yellow connector). The other interactions are described in the text.

BDV N and P interact with the Cdc2-Cyclin B1 complex (Planz *et al.*, 2003), which is a crucial part of the cell cycle (Castedo *et al.*, 2002). In the G_2 phase of the cell cycle, the Cdc2-Cyclin B1 complex is acted on by a series of phosphorylations and de-phosphorylations, resulting in nuclear translocation, which is important for the cells to enter the M phase (Castedo *et al.*, 2002). BDV N interacts with both phosphorylated and non-phosphorylated Cdc2, as well as with Cyclin B1, whereas BDV P interacts only with non-phosphorylated Cdc2 (Planz *et al.*, 2003). Cells transfected with BDV N, had a reduced proliferation rate, indicating an interference of the G_2 to M phase transition. This effect was also seen in BDV-infected cells, but not in cells transfected with BDV P. Thus, BDV interferes with cell proliferation to enable a persistent infection.

As previously discussed, BDV P interacts with TBK-1, and competitively interferes with the phosphorylation of endogenous substrates, resulting in decreased type I IFN-expression (Unterstab *et al.*, 2005). Similar interference occurs in PKCε phosphorylation, where BDV P also acts as a decoy substrate, affecting neuronal plasticity (Prat *et al.*, 2009). BDV P is also suggested to interfere with the normal transport of γ-aminobutyric acid receptors (GABA-R) to the cell membrane, by interacting with GABA-R associated protein (GABARAP) (Figure 9; Peng *et al.,* 2008). This interference could be responsible for causing some of the behavioral changes seen in BDV-infection, since decreased transport of GABA-R to the cell membrane causes anxiety and other behavioral changes (Crestani *et al.*, 1999). Transgenic mice expressing BDV P have neurological signs similar to BDV-infection (Kamitani *et al.*, 2003), implicating direct disease-causing actions of P, possibly by interfering with the GABAergic neurotransmission.

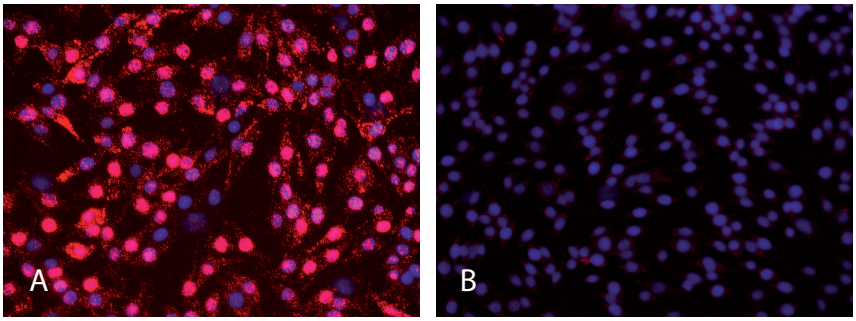

Figure 9. Interaction between BDV P (A) or N (B), and GABARAP in BDV-infected rat astrocytes. To visualize protein-protein interactions, *in situ* PLA was performed. Red staining indicates BDV-GABARAP interactions, and blue staining is the nuclei. Photos: Karl-Johan Leuchowius & Jonas J. Wensman.

BDV P also interacts with a nuclear protein, called high-mobility group box-1 (HMGB1) (Kamitani *et al.*, 2001). HMGB1 is involved in several cellular functions, such as transcriptional regulation (Ueda & Yoshida, 2010), DNA repair (Liu *et al.*, 2010), cell migration (Rauvala & Rouhiainen, 2010) and neurite outgrowth (Rauvala *et al.*, 2000). In BDV-infected cells and in cells treated with BDV P, neurite outgrowth is impaired, most likely because of decreased secretion of HMGB1, as the result of binding with BDV P (Kamitani *et al.*, 2001).

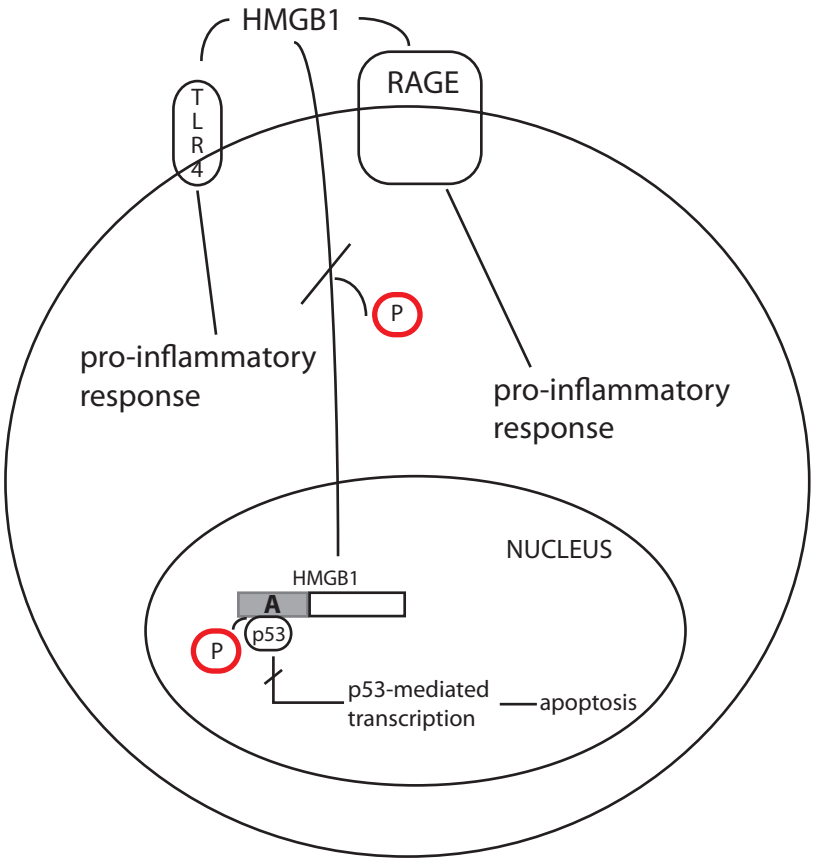

Figure 10. BDV P interferes with the functions of HMGB1. In the nucleus, BDV P outcompetes the binding of p53 to the A-box of HMGB1, thereby inhibiting the p53-mediated transcription, which can lead to apoptosis. Upon infection or cell damage, HMGB1 can be released, and bind to the cellular surface receptors TLR4 and RAGE, in turn inducing a pro-inflammatory response. BDV P reduces the release of HMGB1, and hence its pro-inflammatory actions.

Upon infection or tissue damage, HMGB1 can be released and act as an alarmin, by binding to receptors at the cellular surface (TLR4 and RAGE), inducing a pro-inflammatory response (Yang *et al.*, 2010). In BDV-infected cells, RAGE mRNA expression is decreased compared to non-infected cells, further strengthening the data on interference of normal HMGB1 function by BDV P (Kamitani *et al.*, 2001). Moreover, BDV P outcompetes the binding between HMGB1 and p53, resulting in impaired p53-mediated transcription (Figure 10; Zhang *et al.*, 2003). This interference could be another way for BDV to avoid apoptosis, since p53 is known to induce apoptosis of infected cells (Barber, 2001). Thus, BDV P interferes

with several antiviral, and other, actions of HMGB1 by binding to its p53-binding site, and reducing its release from the cell.

Epidemiology of Bornavirus Infections

For a long time, BD was considered as a disease of horses and sheep in endemic regions of Germany, Switzerland, Lichtenstein, and Austria (Ludwig & Bode, 2000). However, in the 1990's several reports of cases outside these endemic regions, as well as in a broader host range, were published. These findings were preceded by the demonstration of BDV-specific antibodies, antigens and RNA in humans from 1985 and onwards (Bode & Ludwig, 2003; Carbone, 2001; Ludwig & Bode, 2000; Rott *et al.*, 1985), which led to an increasing interest in BDV research. This section will focus on the epidemiology of BDV-infection in animals.

Horses with BD have been shown to shed infectious viruses in nasal and lacrimal fluids (Richt *et al.*, 2000). In addition, sub-clinically infected horses and sheep that are sero-positive can be PCR-positive in these body fluids, as well as in saliva (Vahlenkamp *et al.*, 2002; Richt *et al.*, 1993), indicating a potential shedding of virus. Hence, BDV could spread between animals either by direct or indirect contact. However, this mode of spread is likely not the most important, because only sporadic cases are shown in the same stable of horses (Richt *et al.*, 2000; Ludwig & Kao, 1988). Similarly, staggering disease in cats is mostly seen in only one cat in households with several cats (Berg *et al.*, 1998a). Contradictory data concerning vertical transmission of BDV in horses exist (Hagiwara *et al.*, 2000; Richt *et al.*, 2000).

In horses and sheep, cases of BD are observed in a seasonal pattern, where most cases are presented in late spring to early summer (Dürrwald *et al.*, 2006; Richt *et al.*, 2000; Dürrwald & Ludwig, 1997; Ludwig *et al.*, 1985; Schmidt, 1912). A similar seasonal distribution is also seen in cats (Lundgren, 1992). Annual differences in disease incidence are also present in these species (Wensman *et al.*, 2008; Dürrwald *et al.*, 2006; Ludwig *et al.*, 1985). Together with the historically restricted endemic regions in BD of horses and sheep (Ludwig & Bode, 2000), as well as of cats (Wensman, 2008; Lundgren, 1992; Kronevi *et al.*, 1974) (Figure 11), natural BDV reservoirs have been discussed (Dürrwald *et al.*, 2006; Staeheli *et al.*, 2000; Lundgren, 1995).

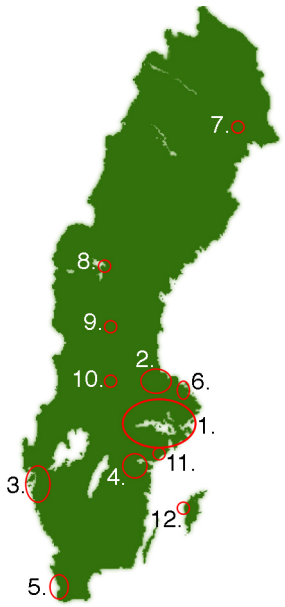

Figure 11. Map of Sweden showing regions where the clinical diagnosis "staggering disease" have been made during the years 1998-2008, based on data from the Agria Animal Insurance Company. The size of the encircled areas is approximately proportional to the number of cases reported. In total, 92 cases were reported, with 65% coming from the previously reported endemic region (1, Areas around Lake Mälaren). In regions previously considered free from disease, cases have also been reported, like the west coast (3) and southern parts (5) of Sweden. Numbers 7-12 represent individual cases, spread over the country. From Wensman (2008).

Wildlife Reservoirs

Because of the persistent infection in experimentally infected rats and mice, resulting in either mild behavioral changes or in sub-clinical infection only (Ludwig *et al.*, 1985), rodents have been proposed as a natural reservoir of BD (Dürrwald *et al.*, 2006; Staeheli *et al.*, 2000). Experimentally infected rodents can transmit BDV both by close contact (horizontal transmission) (Sauder & Staeheli, 2003) and from mother to fetus (vertical transmission) (Okamoto *et al.*, 2003). Hence, a persistent infection could be maintained within a rodent population in nature, and spread BDV to domestic animals through shedding of infectious viruses. In cats, it is mainly those with outdoor access, hunting rodents, that are at higher risk of BDV-infection (Berg *et al.*, 1998a).

Interestingly, recent studies in Finland have shown the presence of BDV-specific antibodies in wild rodents, namely different kind of voles (Kinnunen *et al.*, 2007). Upon experimental BDV-infection, bank voles did not develop pathological alterations in the CNS, despite the fact that BDV-RNA and BDV-antigen were found both in the CNS and in peripheral neural ganglia (Kinnunen *et al.,* 2011). Most of the voles did not show any clinical signs, but some of them presented with hyperactivity or other

neurological signs. BDV-RNA was detected in the feces and urine, indicating potential shedding of infectious virus. Thus, voles could be a wildlife reservoir, and an infection within the population maintained persistence by viral shedding in feces and urine. At least in rats, BDV is transmitted only upon close contact for at least 24 hours, most likely by infectious viruses shed in urine (Sauder & Staeheli, 2003). Thereby, if BDV is transmitted to domestic animals, such as horses and sheep, when infected rodents are contaminating their feed, this contamination probably needs to be intensive and repeated. However, in cats the transmission most likely takes place when infected rodents, such as voles, are preyed upon. Whether the behavioral changes that sometimes can be seen in infected voles (Kinnunen *et al.,* 2011) affect the possibility for these animals to be a prey is not known. However, it is known that the parasite *Toxoplasma gondii* (Apicomplexa) can change the behavior of the intermediate host (rodents) to become an easier prey for the main host, the cat (Webster, 2007).

Another proposed reservoir is wild birds. So far, the only finding of BDV reported from wild birds comes from Sweden, where BDV-RNA was detected in feces of mallards and jackdaws (Berg *et al.*, 2001). Even though the BDV sequences isolated from these birds were similar to other known reference strains and isolates, some differences were seen (Wensman *et al.*, 2008; Berg *et al.*, 2001). Recent findings of ABV have raised the question of whether the isolates found in wild birds are more closely related to the new ABV than previously known. Interestingly, experimental ABV infection in mallards does not result in any clinical signs or pathological lesions (Hoppes *et al.*, 2010), although feces from infected animals is intermittently PCR-positive, and antibodies can be found. Furthermore, ABV can be transmitted by the fecal-oral route to mallards, which accidentally happened in non-infected control mallards when they were kept together with a group of sub-clinically infected cockatiels (Hoppes *et al.*, 2010). Most recently, Canada geese and trumpeter swans with non-suppurative CNS inflammation were found to be ABV-positive, carrying a new distinct genotype of ABV (Delnatte *et al.*, 2011).

Mallards, as well as other migratory birds, are well-known carriers of other viruses, such as influenza virus, West Nile virus and Newcastle disease virus. The migration route of mallards in Northern Europe goes from parts of Germany, where BD is considered to be endemic, to Northern Siberia, passing over Sweden. Hence, wild birds such as mallards, could be important for transporting BDV from endemic regions to previously non-endemic areas.

Ticks have also been considered to be a possible reservoir, since approximately the same regions endemic for BDV are endemic for tick-borne encephalitis virus. However, ticks are probably only mechanical or accidental vectors, because BDV does not seem to replicate in ticks (Schindler, 2004).

In BDV-endemic regions of Central Europe, bi-colored white-toothed shrews have been found to carry BDV (Hilbe *et al.*, 2006). These animals do not show any obvious clinical signs. They harbor BDV in many different tissues, indicating a persistent infection as in rodents (Puorger *et al.*, 2010; Hilbe *et al.*, 2006). Thereby, this insectivore could be another potential reservoir of BDV in certain parts of Europe. However, neither this particular shrew nor any of its closest relatives have their habitat in Sweden.

Other species have also been found to carry BDV or BDV-specific antibodies without showing obvious clinical signs, or with unknown clinical status. In Japan, macaques (12%) and raccoons (2%) have been shown to be seropositive (Hagiwara *et al.*, 2009; Hagiwara *et al.*, 2008). Furthermore, in a few animals of both species BDV-RNA could be found. In France, BDV-RNA has been found in four brain samples from red foxes of unknown clinical status (Dauphin *et al.*, 2001). Whether these findings describe sub-clinical persistent infections or animals with clinical disease, and the potential for these species as reservoirs for BDV, remains unclear.

If there is a wildlife reservoir for BDV, there is obviously not just one, but most likely there are various reservoirs in different parts of the world. Based on the estimated incubation times, from several weeks in sheep (Ludwig & Kao, 1988; Heinig, 1969) up to six months in horses (Mayr & Danner, 1974), and the reported seasonal distribution of BD cases, natural infection can actually occur all year around in these species. The incubation time in cats is not known, but upon experimental intra-cerebral infection it takes three weeks up to 2.5 months for clinical signs to develop (Lundgren *et al.*, 1997). If these experimental data reflect the true incubation time, natural infection should occur mainly from September to April. The mode of spread of BDV therefore most likely varies between different domesticated species, depending on their feeding preferences and how they are housed.

Diagnostics of BDV-Infection

Ante-Mortem Diagnostics

Due to the nature of any persistent infection within the CNS, it is difficult to find specific BDV-markers, such as antigens, antibodies or RNA, in a

living animal with suspected disease. BD is therefore mainly a tentative diagnosis in different animals, made by ruling out other possible explanations for the clinical signs. Even though the clinical signs are characteristic, they are not specific for BDV-infection.

Serology is potentially an aid for the clinician, but in horses with BD the antibodies are not always found in serum, and clinically healthy horses can carry antibodies (Richt *et al.*, 2000; Ludwig *et al.*, 1985). Similar observations have been made in cats. Naturally infected cats developed low or no titers of antibodies, whereas experimentally infected cats developed high titers (Johansson *et al.*, 2002). Other studies have shown antibodies in clinically healthy cats (Helps *et al.*, 2001; Ouchi *et al.*, 2001). Depending on the sensitivity and specificity of the assay used, the sero-prevalence differs even in the same geographical region. The indirect immunofluorescence assay (IFA) is used most commonly (Herzog & Rott, 1980; Ludwig *et al.*, 1973). The sero-prevalence of BDV in endemic regions is around 20% using this method (Richt *et al.*, 2000), and lower in non-endemic areas. In the past decade, enzyme-linked immunosorbent assays (ELISA) detecting antigens, antibodies or circulating immune complexes (CIC) have been developed, which seem to have higher sensitivity (Bode *et al.*, 2001). The sero-prevalence of BDV has been reported as high as 60% in endemic regions using these assays (Ludwig, 2008; Ludwig & Bode, 2000). This could indicate that there is no need for reservoirs for the viral spread (Bode, 2008). There seems to be high cross-reactivity between different viral strains, as IFA using cells infected with an equine BDV strain detected high titers of antibodies in psittacine birds infected with ABV (Herzog *et al.*, 2010), indicating a broad detection range using this classical method for antibody detection.

Antibody detection in cerebrospinal fluid (CSF) is considered to be highly specific for BDV-infection, because antibodies have not been found in healthy horses (Richt *et al.*, 2000; Ludwig & Thein, 1977).

Molecular biological assays for detection of viral nucleic acids (RNA) in clinical samples have been widely used, especially for blood samples (Vahlenkamp *et al.*, 2002; Vahlenkamp *et al.*, 2000; Berg *et al.*, 1999a; Hagiwara *et al.*, 1997; Nakamura *et al.*, 1996; Nakamura *et al.*, 1995). Even though viral RNA has been found in PBMCs and in body fluids from horses and sheep with clinical disease (Vahlenkamp *et al.*, 2000; Richt *et al.*, 1993), this is only for a limited number of cases. Due to the limitations of RT-PCR, only those variants of BDV with sequence similarities to the primers of the assay will be detected. BDV is considered to have a high degree of genetic conservation, but a more divergent strain has been found

(Nowotny *et al.*, 2000). The recent findings of the even more divergent ABV (Honkavuori *et al.*, 2008; Kistler *et al.*, 2008), could indicate higher divergence than previously considered. Hence, the different RT-PCR assays developed so far could fail to detect viral RNA in several cases, due to sequence dissimilarities.

Concerns have been raised about the high risk of contamination when using sensitive assays such as RT-PCR and nested RT-PCR (Dürrwald *et al.*, 2006). This contamination risk is not unique for BDV, and holds true for all RT-PCR and nested RT-PCR assays, and can be reduced by the use of real-time RT-PCR (rRT-PCR) without losing sensitivity (Belák *et al.*, 2009; Schindler *et al.*, 2007; Belák & Thorén, 2001).

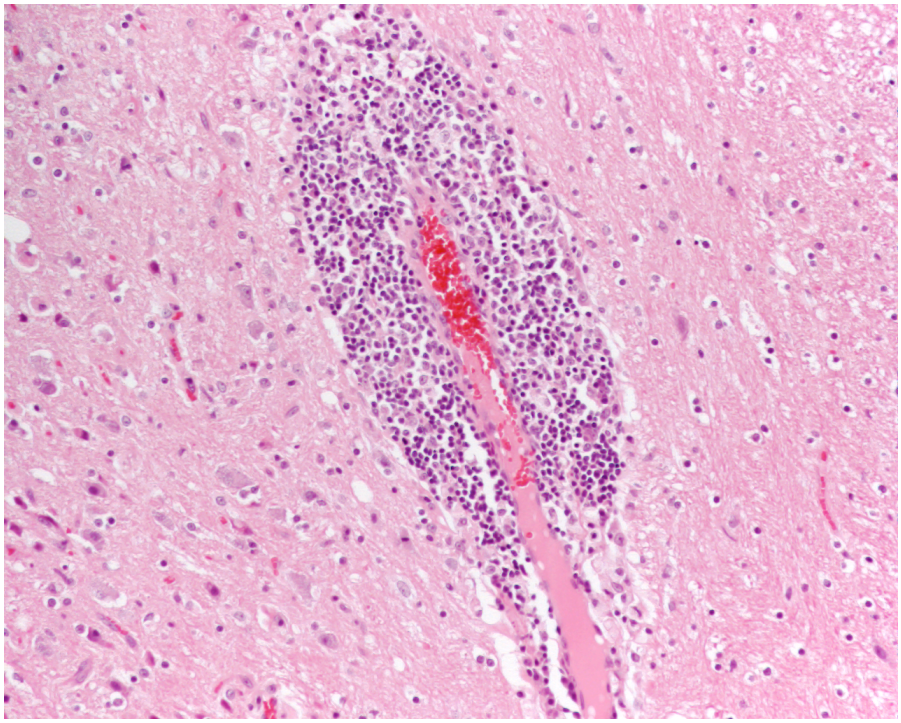

Figure 12. Perivascular cuff consisting of mononuclear cells is one characteristic pathological lesion in BDV-infection. The section comes from a cat suffering from feline BD. Apart from perivascular cuffs, lymphocytic infiltration is also seen in the brain parenchyma. Hematoxylin-Eosin stain. Magnification: lens x20. Photo: Gete Hestvik.

Post-Mortem Diagnostics

BDV-infection is confirmed at necropsy, based on the pathognomonic intra-nuclear inclusion bodies in neurons, named after Joest and Degen who discovered them (Joest & Degen, 1911), and/or the presence of a non-

purulent inflammation of typical regions of the CNS (Figure 12), olfactory bulb, gray matter of the brain stem, basal ganglia and hippocampus (Lundgren, 1992; Gosztonyi & Ludwig, 1984; Joest & Degen, 1911). The presence of BDV-antigen and/or -RNA *in situ* further confirms the correct diagnosis. However, in some species, such as cats, the viral load seems to be lower compared to horses, since the staining by IHC is commonly weaker (Lundgren *et al.*, 1995a).

Antigen detection (Ludwig & Becht, 1977) and/or nucleic acid detection (Zimmermann *et al.*, 1994b) in brain homogenates further confirm the infection.

Humans and BDV

The intriguing results of experimental infection in the lower primate tree shrew (Sprankel *et al.*, 1978), resulting in different behavioral changes, which were also seen later in rats infected as new-borns (reviewed in Briese *et al.,* 1999), led to the question whether BDV could be involved in human disease. The first serological evidence of human BDV-infection was discovered in patients with affective disorders, whereas the control subjects without signs or history of these disorders were all negative (Rott *et al.*, 1985).

This finding led to worldwide efforts to study similar and other patient groups, using serological techniques, staining for antigen and RNA in tissues, and molecular methods (Bode & Ludwig, 2003; Carbone, 2001). By these methods, BDV was detected in brain tissues of humans with hippocampal sclerosis (Czygan *et al.*, 1999; de la Torre *et al.*, 1996). The first human BDV-variant was isolated in cell cultures and in laboratory animals from brain tissue of a patient with schizophrenia, and sequence analysis showed genetic similarities to other isolated BDV-strains (Nakamura *et al.*, 2000). BDV has also been considered a cause of viral encephalitis (Li *et al.*, 2009).

Because of the close genetic relationship of most BDV-isolates in humans as well as in animals, false positive results due to laboratory contamination have been discussed (Dürrwald *et al.*, 2007). Recent findings show that BDV-like elements have been incorporated into the genome of humans, and other mammals (Belyi *et al.*, 2010; Horie *et al.*, 2010). Whether these data support the reports of BDV-infection in humans, or could influence results based on molecular biological detection methods, is an open question.

Treatment and Prophylaxis

During the 19th and early 20th century, several herbal and medical treatments were employed on horses with BD, but none of them seemed to work (Zwick, 1939; Nicolau & Galloway, 1928; Schmidt, 1912). More recently, anti-viral drugs have been investigated for their potential inhibition of viral replication in infected cell cultures, and in experimentally infected animals. Ribavirin has been promising in cell cultures (Jordan *et al.*, 1999; Mizutani *et al.*, 1998) and in experimentally infected animals (Lee *et al.*, 2008; Solbrig *et al.*, 2002), partly by facilitating the non-cytolytic viral clearance of the host immune response (Solbrig *et al.*, 2002). However, its use in naturally infected animals remains unknown. In addition to *in vitro* studies, another drug, amantadine, has been used for treatment of natural infection in animals and humans (Dieckhöfer, 2008; Dietrich & Bode, 2008; Ohlmeier *et al.*, 2008; Dieckhöfer *et al.*, 2004; Bode *et al.*, 1997), though its antiviral effects on BDV in cell cultures have been questioned (Cubitt & de la Torre, 1997; Hallensleben *et al.*, 1997).

Cats with feline BD have been commonly treated with corticosteroids, to reduce the inflammatory response, and the clinical signs this response lead to. This treatment seems to be beneficial when used in the early stage of disease (Wensman *et al.*, 2011; Berg, 1999), though the use of immunosuppressive treatment could lead to increased virus replication.

Early work by Zwick and his coworkers showed that it was possible to immunize horses, preventing clinical disease upon viral challenge (Zwick *et al.*, 1928). Therefore, vaccination occurred in Germany from the 1920's onwards, though it was recommended only in endemic regions (Heinig, 1969). In the former Federal Republic of Germany (West Germany), the efficacy of the vaccine was questioned, and vaccinations ended in the late 1970's (Dürrwald *et al.*, 2006). The vaccinations in the former German Democratic Republic (East Germany) did not come to an end until the reunification of Germany. The spread of BDV due to vaccination with live vaccine strains has been suspected (Ludwig & Bode, 2000), though according to molecular epidemiological data this spread seems to have been limited (Dürrwald *et al.*, 2006). Today there is no medical prophylaxis in use to prevent BDV infection, though isolation of sick animals and hygienic safety measures can reduce the spread of disease (Ludwig & Bode, 2000).

Aims of the Thesis

The overall aims of this thesis were to improve current detection methods, and to increase knowledge about BDV-host interactions, especially in naturally infected animals.

The specific aims were to

- Develop novel methods for improved detection of BDV infection markers (RNA, antigen, and antibodies; Papers I, II & III);
- Apply and evaluate these methods in cats with BD to improve the current *ante-mortem* diagnostics (Paper II);
- Study BDV-host protein-protein interactions in infected animals (Paper III);
- Study the host immune response in feline BD (Paper IV) and its possible implication for development of clinical signs (Paper II).

Materials and Methods

This section will briefly discuss and comment on the materials and methods used in the studies of this thesis. More detailed descriptions are provided within each individual paper.

Sampling

An essential part of virus detection, as well as gene expression analyses, is sampling, and sample handling. Brain tissue samples from cats with or without signs of BDV-infection were collected at necropsy (Papers I, II & IV). For all cats studied in Paper II and for some of the cats used in Paper IV, necropsy was performed in close connection with euthanasia. However, in Paper I and in the majority of the cases in Paper IV, the time from euthanasia to necropsy and sampling was probably around 24 h. During this time, the carcasses had been stored at c. +4°C. In most cases, tissue samples were not snap-frozen, but just put immediately into a deep freezer (c. -70°C). Similar handling is likely for samples taken at the clinics (Paper II).

Some samples had been exposed to repeated thawing and freezing cycles. These handling procedures probably negatively influence the outcome of both virus detection and gene expression analyses. As discussed below, for gene expression analyses, this can be overcome by using reference genes when normalizing the data.

RNA-Extraction

RNA is known to be sensitive to degradation by ribonucleases (RNase), which are present everywhere. It is therefore important to reduce potential exposure to RNase, for example by using RNase-free reagents and plastics,

and to reduce the possibility for RNase to be active, for example by keeping the RNA at a low temperature for as long as possible.

In the studies presented in this thesis, total RNA was extracted by a phenol and guanidine isothiocyanate solution (TRIzol or QIAzol reagent, Invitrogen or Qiagen, respectively), in combination with the addition of chloroform, according to the manufacturer's recommendations. A combination of this technique and on-column RNA isolation (Qiagen), sometimes with a DNase treatment step, was used to improve the quality of the RNA (Papers II & IV).

Regardless of extraction method, a most crucial step is homogenization of tissues. For brain tissues, homogenization is best performed in TRIzol or QIAzol. As BDV causes a persistent infection within the CNS of cats, this virus is only present at low levels. The presence of the virus is also brain region specific. Even if the correct brain region is chosen, there could be differences in viral load within this region. Only a small piece of brain is used for each RNA-extraction (approx. 0.5 cm^3), hence, there could be false negatives because of picking the "wrong" piece.

If the RNA is eluted in too high a volume, the viral RNA could be diluted below the limit of detection. This dilution effect could be the explanation for why manually extracted RNA from blood samples was positive (Paper II) while robot extracted RNA was negative (data not shown).

cDNA-Synthesis

Synthesis of cDNA or reverse transcription (RT) can be performed in several ways. In Paper I, cDNA-synthesis was included as the initial step in a one-step rRT-PCR using the same enzyme and primers as the following PCR. This approach decreases the time of handling, and the possibility for contamination due to multiple opening of tubes and during pipetting.

Synthesis of cDNA for BDV detection was performed either in a one-step rRT-PCR or as a separate step (two-step rRT-PCR; Papers II & IV). In the two-step approach, gene-specific primers were used (Berg *et al.*, 2001), together with Superscript III (Invitrogen), as this approach was shown to have higher sensitivity, compared to random hexamers as cDNA-primers or the use of commercial RT-primers (Qiagen). However, there is a risk of false negative results when using gene-specific primers for cDNA-synthesis, as these primers could miss more divergent strains.

Oligo(dT)-primers specific for the poly-A tail of mRNA were used for gene expression analyses (Paper IV). As some genes are intron-less, like

IFN-α and -β, it is important to carry out a –RT reaction (without RT enzyme) as a control for genomic DNA contamination. This approach is also important when primer and probe design are not performed to cover an intron-exon junction, thereby decreasing the possibility of contaminating genomic DNA contributing to the amplification of a PCR product. In my experience, this technical control is crucial for all gene expression analyses, at least in an initial optimization of (r)RT-PCR assays.

Real-Time RT-PCR

Real-time or quantitative RT-PCR has revolutionized gene expression analysis and molecular diagnostics. This method is easy to automate by the use of pipetting robots, thereby increasing speed and decreasing the need for laboratory personnel. Furthermore, the possibility for absolute or relative quantification of gene expression has made rRT-PCR the gold standard for confirmation of global gene expression analysis (microarray data).

Several different chemistries are available for rRT-PCR. In the studies presented in Papers I, II & IV, the TaqMan-probe chemistry was used. The TaqMan-probe is an oligonucleotide, complementary to the target sequence, labeled with a reporter dye and a quencher. In Paper I 6-carboxyfluorescein (FAM) and 6-carboxy-X-rhodamine (ROX) were used as reporter dyes, and in Papers II & IV FAM was used. In all TaqMan-based assays (Papers I, II & IV), black hole quencher (BHQ) was used. BHQ has the advantage of emitting heat instead of light upon excitation, thereby decreasing possible background signals.

TaqMan-based assays are gene-specific, while SYBR-green is an intercalating dye, binding non-specifically to any double-stranded nucleic acid (RNA/DNA). The latter chemistry was used for the rRT-PCR of reference genes (Paper IV).

Primer and Probe Design

The design of primers and probes is an important step for every PCR, because primers and probes determine the specificity of the assay. This design can be done using different software, though manual design is still valid. If multiple variants of the target sequence are aligned, the use of software will facilitate the design, especially if degenerate sites in the primers are allowed.

If 5'-AT rich overhangs are added to the primers then the sensitivity of the rRT-PCR assay has been shown to increase (Afonina *et al.*, 2007). The BDV P and L primers (Paper I), as well as the BDV N primers (Schindler *et*

al., 2007), were modified in this way to see if this possibility was true also in these systems. For BDV P, the achieved fluorescence was higher, though C_T-values remained similar, thereby giving a more secure signal in dubious samples. However, in the BDV L assay, this effect was not as obvious, and for BDV N the original primers were better than the modified ones. Most likely, 5'-AT rich overhangs are more important if the primers used are degenerate, as in the case of BDV P and L. When consensus primers are used, as in the case of BDV N, modifying the primers is not beneficial.

Determination of Sensitivity

The sensitivity of rRT-PCR is usually superior to that of conventional RT-PCR, and is c. 1-100 copies of the targeted gene or viral genome per reaction. This level of sensitivity is similar to that of nested RT-PCR, but with the advantage of not handling PCR-products as templates for the second round of PCR (Belák *et al.*, 2009).

However, analytical sensitivity (determination of the lowest virus level needed for detection) is just an approximation, because it is determined by calculating number of copies of a plasmid or *in vitro* translated RNA based on the DNA/RNA concentration and molecular weight of the nucleic acid. Then, a dilution series is made according to these calculations. Hence, there are several approximations, as well as possibilities for pipetting errors when determining the analytical sensitivity.

The diagnostic sensitivity is determined by using a number of known infected samples (Belák & Thorén, 2001). However, this determination relies on a second assay for detection of infection, which is difficult in BDV-infection. In the studies of this thesis, typical clinical signs and characteristic pathological lesions have been used as a "gold standard" for BDV-infection (Papers I, II & IV).

In situ PLA

Until recently, studies of protein-protein interactions *in situ* in tissues or in genetically unmodified cells have been difficult. A novel and selective tool to study individual proteins or protein-protein interactions, the *in situ* proximity ligation assay (PLA), was developed to meet this need (Söderberg *et al.*, 2006), and is now available commercially (Duolink, Olink Bioscience).

In situ PLA was used for detection of BDV-proteins, as well as for BDV-host protein-protein interactions in persistently infected cells and brain tissues from experimentally and naturally infected animals (Paper III). At

first, fluorescently labeled detection oligonucleotides were used also for tissue samples (Figure 13), but because of a high degree of auto-fluorescence in brain tissues the fluorescence was converted to chromogenic signals by DuoCISH (Dako).

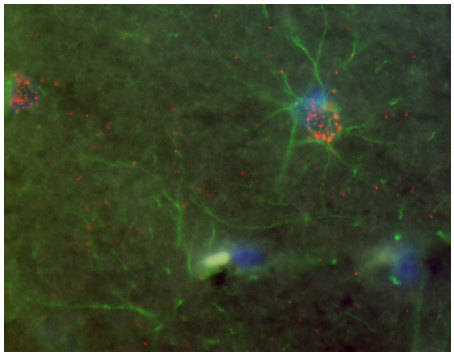

Figure 13. BDV P *in situ* PLA of brain tissue from an experimentally infected cat. BDV P is labeled red, glial fibrillary acidic protein (GFAP) as a marker for glial cells is labeled green, and cell nuclei are labeled blue. There is a high degree of auto-fluorescence, especially in the green filter. Photo: Karl-Johan Leuchowius.

Antibody Selection

For all antibody-dependent methods, antibody selection is crucial. In veterinary medicine there is usually a lack of reagents suitable for the species of interest, especially if several different species are to be studied. For the studies in Paper III, commercial antibodies for detection of different cellular proteins were purchased (Abcam). The antibodies were chosen for their broad species recognition and for their proven usage in IHC and/or IFA. Initially, the antibodies were evaluated by IFA, where antibody titrations were performed. Then, the antibodies were used in the *in situ* PLA in cells, to detect potential BDV-host protein-protein interactions. For tissues, an initial antibody evaluation was performed by IHC, followed by *in situ* PLA.

Gene Expression Analysis

As previously discussed, rRT-PCR has become widely used for gene expression analysis and for confirmation of microarray data. However, mRNA expression does not necessarily lead to translation into a protein. Therefore, protein expression and activity analysis is further needed, in order to determine whether an increase or decrease in gene expression is biologically relevant.

Moreover, for some genes that are constitutively expressed and/or key regulators, a small increase or decrease could lead to a high biological impact.

Reference Genes

As the quality of tissues, and hence also the quality of the RNA, used in Paper IV was affected by sample handling, especially different time from euthanasia to necropsy, sampling, and freezing of samples, the use of reference genes was essential. The RNA quality is not essential in gene expression analysis by rRT-PCR, because the PCR-products are usually relatively short (<250 bp), thus the results are less affected by RNA degradation (Nolan *et al.*, 2006). However, the use of reference genes is needed to compensate for imperfect RNA quality.

Moreover, it is most important to use more than one reference gene, especially when handling samples taken at necropsy (Nolan *et al.*, 2006). It is also crucial to select the right reference genes for the tissue of interest, based on the stability of expression (Vandesompele *et al.*, 2002). In Paper IV, previously validated feline reference genes were used (Penning *et al.*, 2007); however, brain tissue specific selection, based on expression stability, was performed. Reference genes, where genomic DNA was substantially amplified, were avoided.

Normalization of Data

There are several ways to normalize rRT-PCR data. In Paper IV, the individual C_T-values of the gene of interest, as well as the reference genes, were inverted, in order to interpret the obtained data more easily (Jiwakanon *et al.*, 2010). Lower C_T-values mean higher gene expression, and vice versa. Without inverting the C_T-values, an increase in gene expression is therefore seen as a lowered value, which renders the data more difficult to interpret.

For normalization, the average of three reference genes was used for each individual sample, which should give the normalized data more validity. In previous feline gene expression analyses, mostly one reference gene has been used for normalization (Penning *et al.*, 2007).

Results and Discussion

Papers I, II & IV – Detection of BDV-Infection Markers in Cats

The safe diagnosis of feline BD is hampered by a lack of fully reliable diagnostic methods for the confirmation of BDV-infection in the live cats. For the clinician, staggering disease or feline BD is therefore a tentative diagnosis made by ruling out other possible explanations for the clinical signs. Previous studies have detected BDV-RNA in different body fluids of infected horses and sheep (Vahlenkamp *et al.*, 2002; Berg *et al.*, 1999a; Richt *et al.*, 1993), suggesting rRT-PCR as one way of confirming infection. Serological methods have been widely used for a long time in several species (Bode *et al.*, 2001; Lundgren & Ludwig, 1993; Herzog & Rott, 1980; Ludwig & Thein, 1977; Ludwig *et al.*, 1973), though cats are thought to develop only low or non-detectable antibody titers upon natural BDV-infection (Johansson *et al.*, 2002). Moreover, antibodies have been detected in healthy cats, and the prevalence in the normal cat population is unknown, which makes it even more difficult to interpret serological results. For this reason, an rRT-PCR was developed (Paper I), and evaluated for its clinical diagnostic value (Papers II & IV). BDV-serology was established and evaluated, with special regard to antibody prevalence in a reference cat population (Paper II).

Detection of BDV-RNA

An rRT-PCR assay was developed for simultaneous detection of BDV P and L genes, either in a one-tube (duplex) or a two-tube (simplex) format (Paper I). Primers and probes for BDV P and L were designed by aligning several BDV-strains, including the most divergent strain known at that time, BDV No/98 (Pleschka *et al.*, 2001; Nowotny *et al.*, 2000), to have as broad an assay as possible. All reference strains of BDV (strains V, He/80, and

No/98) were detected, as well as five field strains from brain tissues of naturally infected cats, and a feline isolate previously used in experimental infection of cats (Lundgren *et al.*, 1997; Lundgren *et al.*, 1995b). As expected, non-BDV viruses used in the specificity test were not detected.

The assays had high analytical sensitivity: down to 10 copies per reaction could be detected in the two-tube format of both targeted genes, and in the one-tube format of BDV P. In the one-tube format of BDV L, the analytical sensitivity was 10-fold decreased, so that approximately 100 copies per reaction were detected. This decrease could be due to primer-probe competition, often seen in multiplex PCR-assays. The BDV L primers had several degenerate sites. Therefore, there is not only a set of two primers in this assay, but a mixture of several variants of the different primers, which could further increase the possibility for competition.

In the analysis of field strains, BDV L was detected in four out of five samples, but only at very low levels, whereas BDV P was easily detected in all samples (Paper I). Hence, BDV L is not as suitable for diagnostic purposes as BDV P. This system was therefore not used in the further studies (Papers II & IV). BDV N and P are the most abundant genes, based on the 3' – 5' decrease in molarity of gene transcripts common to all viruses of the order *Mononegavirales*. Therefore, an rRT-PCR for BDV N was also used (Schindler *et al.*, 2007) to increase the likelihood of detection (Paper II). The BDV P rRT-PCR was modified to increase the sensitivity (Papers II & IV; Afonina *et al.,* 2007).

In the study presented in Paper II, cats fulfilling the clinical inclusion criteria (gait disturbances with or without behavioral changes) and having characteristic pathological lesions were sampled at the clinic and at necropsy for detection of BDV-RNA. In eleven of 19 cats (58%), BDV-RNA was detected in at least one sample, taken both at the clinic (N=6), and at necropsy (N=6). In two cats, multiple samples, taken both at the clinic and at necropsy, were positive for BDV-RNA. Most commonly, BDV-RNA was detected in blood (N=5), and olfactory epithelium (N=4), but BDV-RNA was not detected in nasal fluids, as has been found in horses and sheep (Vahlenkamp *et al.*, 2002; Richt *et al.*, 1993). As the majority of the positive blood samples (3 out of 5) came from cats showing mild neurological signs, rRT-PCR could be helpful for confirmation of the diagnosis in these cases.

Not all cats showing characteristic neurological signs, and typical pathological lesions, of feline BD, were confirmed to be positive by rRT-PCR (Papers II & IV). The explanations could be the low viral load in cats (Lundgren *et al.*, 1995a) compared to for example horses, where BDV-RNA most often can be easily detected by (r)RT-PCR (Dürrwald *et al.*,

2006) and/or sequence dissimilarities. So far, no feline BDV isolate has been completely sequenced, and there are only a few partial sequences available. The difficulty of obtaining (longer) sequences from feline BDV isolates is probably due to the low viral load in cats and/or sequence dissimilarities, in combination with sampling procedure and sample handling. Thus, it is still not known whether some feline strains are more similar to ABV, or if ABV can infect cats, as the rRT-PCR used in these studies probably does not detect the more divergent ABVs.

Detection of BDV-Specific Antibodies

As BDV-RNA was not detected in all cats with feline BD, serological investigation was also performed (Paper II). In this study, a classical method for antibody detection, IFA, was used. This method is routinely used at the National Veterinary Institute (SVA) in Sweden, though not yet for detection of BDV-specific antibodies.

BDV-specific antibodies were detected in serum samples from 13 of 16 tested cats (81%). In the studied reference population at highest risk for exposure of BDV, 16% were positive. This is a similar proportion to that of healthy horses in endemic areas of Germany, using the same method (Richt *et al.*, 2000). The number of cats included in the study is limited (feline BD cases: N=16; high risk reference population: N=25), but if the results reflect the situation in a larger population of cats, the positive prediction value in endemic regions is 76%, and the corresponding negative prediction value is 88%. Three cats with feline BD had antibodies in their CSF. As BDV-specific antibodies are only detected in the CSF of horses with BD (Richt *et al.*, 2000; Ludwig & Thein, 1977), the presence of antibodies in CSF is highly indicative of BDV-infection.

In Paper II, all cats studied had typical clinical signs of feline BD, as well as pathological lesions in concordance with BDV-infection. Feline BD should be high on the list of differential diagnoses when a cat presents signs like ataxia, absent or decreased postural reactions and menace response, and behavioral changes, especially if the cat has outdoor access. Previously, feline BD was considered to be present in Sweden only in the areas around Lake Mälaren (Lundgren, 1992; Kronevi *et al.*, 1974), but lately the disease has been diagnosed in other regions of Sweden as well (Figure 11; Wensman, 2008). Serology would further confirm the clinical suspicion in these cases. However, the situation remains unclear for more questionable cases, as these probably have not fulfilled the inclusion criteria of this study. Whether it is more common for cats with typical signs of feline BD to develop an antibody response is not known, however, it seems unlikely. Further studies

of the prevalence of BDV-specific antibodies in cats with neurological disease are needed, for a better understanding of disease development in feline BD and for better advice to clinicians in making correct diagnosis. These studies should include cats from different regions of Sweden.

Molecular Detection versus Serology

Previous results indicated that naturally infected cats did not develop antibody titers sufficiently high for serological diagnostics (Johansson *et al.*, 2002). Therefore, an rRT-PCR was developed for molecular detection of BDV (Paper I), and applied for diagnostic purposes (Papers II & IV). Even though a majority of the cats were positive for BDV-RNA (58% in Papers II & IV), it was only detected in samples from live animals in a few cases (32%; Paper II). Moreover, there was not a single clinical sample where the material was consistently positive, although blood was the most common one. Possible explanations for the low number of PCR-positive cats could be low viral load, due to the nature of BDV persistence in the CNS, together with RNA degradation during sampling procedure and sample handling, as well as sequence dissimilarities. However, compared to other studies of feline BD (Kamhieh & Flower, 2006) the number of PCR-positive cats was quite high (Papers II & IV), indicating high sensitivity of the developed rRT-PCR. Based on the results in Paper II, the value of rRT-PCR as a diagnostic method to confirm the clinical suspicion of feline BD is questionable, except for cats showing mild neurological signs, where most of the PCR-positive blood samples (3 out of 5) were found.

IFA is a well-established serological method, commonly used to diagnose BD (Herzog & Rott, 1980; Ludwig *et al.*, 1973), even though it is less sensitive than ELISA (Bode *et al.*, 2001). Recent studies have shown that a conventional BDV-IFA, using equine BDV as antigen, detects antibodies developed as a response to ABV infection in psittacine birds (Herzog *et al.*, 2010). These results indicate the validity of IFA even to diagnose infections of more divergent Bornaviruses. As no feline BDV strain has been completely sequenced, and only a few partial sequences have been reported, more divergent variants of feline BDV could exist. These sequence dissimilarities could, as previously discussed, explain the difficulty of diagnosing feline BD by molecular methods. Likewise, it could be one explanation for why antibodies are detected in the absence of BDV-RNA, since antibodies towards divergent strains cross-react with the equine BDV antigen used in the IFA. Six of the cats were only sero-positive, whereas two were only PCR-positive, and seven were positive for both infection markers (Paper II).

Overall, serology is a useful tool to guide the clinician when making the diagnosis of feline BD, especially in cats showing typical clinical signs. In addition, BDV rRT-PCR of blood samples could be helpful when cats are showing mild neurological signs.

Papers II & IV – Immune Response in Feline BD

In Paper IV, IFN-γ mRNA expression was studied in cats with feline BD, and compared to the expression in cats without BDV-infection. As previously discussed, not all cats were confirmed to harbor BDV-RNA in brain tissues. This study clearly showed that IFN-γ is highly expressed in cats with feline BD, regardless of whether BDV-RNA could be detected or not, whereas non-diseased cats expressed no or low levels of IFN-γ mRNA.

Only one cat (cat V, Paper IV) was negative for BDV-infection markers, when samples other than brain tissues used for BDV-RNA detection, as well as serological data, are taken into account (Paper II).

IFN-γ is an important antiviral cytokine of the adaptive immune response, and a key player in viral clearance, especially in sensitive tissues such as the CNS where cytolysis is unwanted (Chesler & Reiss, 2002). Release of IFN-γ not only takes place in acute infection, but can also be prolonged, even after the virus has been cleared (Rottenberg & Kristensson, 2002). Thus, IFN-γ can affect the brain without the presence of the virus. Previous studies show, that IFN-γ can block BDV infection in some cell lines (Sauder *et al.*, 2004), and also block BDV multiplication in tissue slice cultures (Friedl *et al.*, 2004). In experimentally BDV-infected mice, IFN-γ is necessary for viral clearance by CD8+ T cells (Hausmann *et al.*, 2005), and in experimentally BDV-infected rats the IFN-γ inducible protein IP-10 is highly expressed (Jehle *et al.*, 2003; Sauder *et al.*, 2000). Hence, IFN-γ is an important player in the host immune response to BDV-infection.

The importance of IFN-γ in viral clearance can also be an explanation for why not all cats with feline BD are positive for BDV-RNA. It has been previously noted that cats with a high degree of inflammation are less likely to be positive for BDV-RNA by RT-PCR, whereas cats with milder inflammatory reaction are more frequently PCR-positive (Anna-Lena Berg, personal communication). However, BDV is still present in cats expressing IFN-γ, indicating mechanisms for evading this antiviral response by BDV. One such mechanism has been assigned to BDV P, which inhibits inducible nitric oxide synthase (iNOS) expression (Peng *et al.*, 2007), induced by for example IFN-γ.

The low viral load in cats, compared to horses, could indicate a more efficient clearance of BDV in this species. Horses usually have strong immunoreactivity for BDV-antigen, and the antigen expression co-localizes in the same brain regions as inflammatory changes (Gosztonyi & Ludwig, 1984), whereas cats have weaker antigen expression (Lundgren *et al.*, 1995a), with a similar distribution pattern as in horses (Anna-Lena Berg, personal communication). The IFN-γ expression, or the activity of IFN-γ, could be different between species, though this hypothesis needs to be proven by for example comparative gene expression analyses.

Some of the cats studied in Paper IV were also included in Paper II. Interestingly, cats with mild neurological signs had significantly higher IFN-γ mRNA expression compared to cats with severe neurological signs (Papers II & IV). The IFN-γ expression followed the degree of inflammatory reaction: lower IFN-γ expression in cats with milder pathological lesions, and higher IFN-γ expression in cats with severe pathological lesions. Cats with mild neurological signs had moderate-severe pathological lesions, suggesting that a heavy inflammatory reaction does not necessarily lead to more severe clinical signs (Paper II). As BD is considered to be immune-mediated (Stitz *et al.*, 1995), this observation is very interesting, since it indicates a more virus-induced effect in the development of clinical signs in feline BD than previously considered. This is similar to what is seen in experimental infection of newborn rats, where neurological signs, such as behavioral changes, can be seen without inflammation (Gosztonyi & Ludwig, 1995). Further indications of direct viral effects on pathogenesis are seen in transgenic mice, expressing BDV P in glial cells, which induce neurological signs, similar to BDV-infection (Kamitani *et al.*, 2003). Also in one cat, neurological signs were seen without inflammatory reaction, but in the presence of BDV (Berg & Berg, 1998), further suggesting direct effects attributed to BDV. Such direct effects could be BDV-host protein-protein interactions, like the interaction between BDV P and GABARAP, interfering with the trafficking of GABA-R to the cellular membrane, which in turn could cause behavioral changes due to disturbances in the GABA neurotransmission (Peng *et al.*, 2008).

Paper III – Visualization of Phosphorylated BDV P and BDV-Host Protein-Protein Interactions

Several protein-protein interactions between BDV and its host have been reported (Planz *et al.*, 2009). In the study presented in Paper III, two cellular proteins previously demonstrated to interact with BDV proteins were

visualized in cell cultures and brain tissues by the use of *in situ* PLA. Furthermore, *in situ* PLA was also used for demonstration of phosphorylated BDV P, and compared to IHC staining of brain tissues of experimentally and naturally infected animals.

Visualization of Phosphorylated BDV P

Initially, IFAs of persistently BDV-infected C6 cells (C6BDV; rat astrocytoma cell line) and IHCs of brain tissues were performed for BDV P, as well as host proteins, to evaluate the antibodies and protocols. In the IHC for BDV P, a rabbit polyclonal antibody was used (Johansson *et al.*, 2002). Neurons of the infected animals had a strong staining for BDV P, whereas no specific staining was seen in the non-infected controls.

In the *in situ* PLA for BDV P, a set of two primary antibodies was used. In addition to the rabbit polyclonal antibody used in the IHC, one mouse monoclonal antibody (Ludwig *et al.*, 1993) was used. According to epitope mapping, this monoclonal antibody specifically binds to phosphorylated BDV P, further confirmed by determining *in vitro* phosphorylated recombinant BDV P in an antigen ELISA (Bode, 2008). Thus, the *in situ* PLA for BDV P will selectively detect phosphorylated BDV P.

A similar distribution pattern was seen when using both IHC staining and *in situ* PLA, but comparatively fewer BDV-positive cells were seen using *in situ* PLA. This difference could be contributed by the need for dual recognition in the *in situ* PLA, and corresponds to the phosphorylation pattern of BDV P. Upon phosphorylation by PKCε, BDV P down-regulates the viral RNA-dependent RNA-polymerase (L) (Schmid *et al.*, 2007). However, phosphorylated P is important for the efficient spread of BDV (Schmid *et al.*, 2010), and competes with endogenous PKCε substrates, thereby affecting neuronal plasticity (Prat *et al.*, 2009). The phosphorylation of BDV P seems to have dual effects on the viral life cycle: replication control, which could be of importance in persistent infection, and promoting viral dissemination. The exact mechanisms for the latter are not known. Further studies are also needed for investigating any differences in specific distribution of phosphorylated P during different stages of infection, regarding brain regions and cellular compartments.

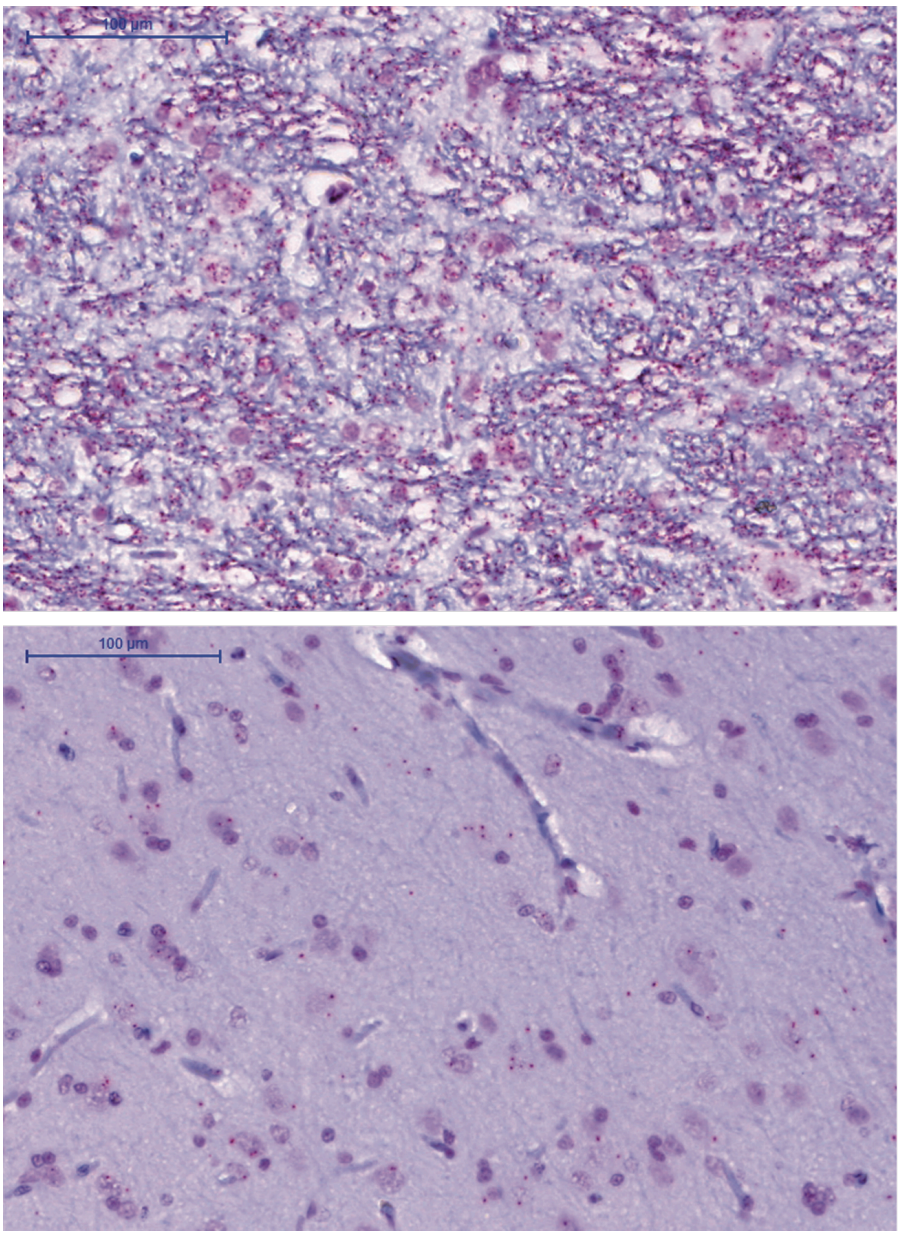

Figure 14. Detection of phosphorylated BDV P in cats using *in situ* PLA. The upper micrograph shows a cat with feline BD (cat D, Paper I), and the lower micrograph shows a non-infected control cat. More signals are seen in the diseased cat compared to the control. Photos: Karl-Johan Leuchowius.

The BDV P *in situ* PLA was also used in cats, and preliminary data shows the presence of phosphorylated BDV P in a cat with feline BD, and a few signals in a cat without this disease (Figure 14). Due to the need for dual recognition, *in situ* PLA is more selective than IHC, and could be a useful tool to increase specificity when specific IHC staining is difficult to distinguish from the background.

Visualization of BDV-Host Protein-Protein Interactions

The BDV-host protein-protein interactions studied in Paper III were the interactions between BDV N/P and Cdc2 (Planz *et al.*, 2003), and BDV P and HMGB1 (Kamitani *et al.*, 2001), previously reported. By the use of *in situ* PLA, these interactions could be visualized initially in C6BDV cells, where the signals most commonly were seen inside the nuclei, and then also in brain tissues of experimentally (Cdc2 and HMGB1) and naturally (Cdc2 only) infected animals.

Previously, both BDV N and P have been demonstrated to interact with Cdc2, although it is N that interferes with the functions of Cdc2 by reducing the proliferation rate in transfected and infected cells (Planz *et al.*, 2003). Therefore, it did not come as a surprise that BDV N, as well as BDV P interactions with Cdc2 could be visualized in BDV-infected cells. For HMGB1, BDV interferes with the multiple functions of this protein through interaction by P and not N (Zhang *et al.*, 2003; Kamitani *et al.*, 2001). On the other hand, BDV N and P interact with each other (Berg *et al.*, 1998b); and thus, BDV N-HMGB1 interactions visualized in C6BDV, most likely originate from indirect interactions via BDV P.

In tissues, BDV N-Cdc2 interactions were detected in scattered neurons, both in an experimentally infected rat and in a naturally infected horse. As this interaction slows down the proliferation rate of cells (Planz *et al.*, 2003), BDV could use this to promote the establishment of a (persistent) infection, especially in dividing cells.

The interaction between HMGB1 and BDV P was detected only in the experimentally infected rat. This rat was sacrificed rather early in infection (30 days post infection), when BDV is likely to be in the process of establishing persistence in the studied regions of the CNS. Probably, the interference of the pro-inflammatory functions of HMGB1 is more important early in the course of infection than at the end-stage, when persistence already is established. The naturally infected horse showed typical signs of BD, loss of weight, ataxia, circling movement, and depression, suggesting a longer duration of infection. HMGB1 is expressed at different levels depending on age, at least in the mouse, where younger

animals have comparatively higher expression (Enokido *et al.*, 2008; Guazzi *et al.*, 2003). The horse included in this study was 12 years old at euthanasia, and the rat was four months old when sacrificed. Even though the rat was adult, the horse should be regarded as comparatively older. From this material it is not possible to draw any conclusions about species differences in BDV P-HMGB1 interactions. Hence, duration of infection, age differences, but also differences in infection route, could be explanations behind the absence of signals in the horse.

Paper III represents the first study where virus-host protein-protein interactions have been visualized *in situ* in infected cell cultures and tissues by the use of *in situ* PLA.

Conclusions

This thesis has focused on BDV and its hosts, by developing different techniques to study presence of infection markers (BDV-RNA, BDV-antigen and BDV-specific antibodies), and by studying BDV-host interactions. Based on these studies, and the experiences gained, the main conclusions are:

- Real-time RT-PCR is a highly sensitive and specific assay for detection of BDV-RNA, as down to 10 copies of the gene targets per reaction can be detected. Three reference strains, including the most divergent strain No/98, as well as five feline field strains, and a feline BDV isolate, were detected, indicating a broad range of detection.

- In most cats with typical clinical signs of feline BD, and with characteristic pathological lesions, at least one BDV-infection marker (BDV-RNA and/or BDV-specific antibodies) could be detected. The most common marker is the presence of antibodies in serum, which were detected in 81% of cats with feline BD, compared to 16% of a reference population at high risk. Serology is therefore the most helpful diagnostic tool when presented with a clinical case of feline BD. In addition, molecular detection of BDV in blood can aid in the confirmation of BD in cats with mild neurological signs.

- *In situ* PLA is a useful tool to study the presence of BDV-antigen, and increases the selectivity by the use of dual recognition. This assay can be particularly useful for *post-mortem* diagnostics when antigens are hard to distinguish from background signals in conventional IHC, thus increasing the need for highly specific detection methods.

- BDV proteins interact with host cellular proteins of infected animals, most likely interfering with important functions in favor of virus persistency.

- Cats with BD express IFN-γ, and this expression is higher in animals showing mild neurological signs, in which moderate-severe pathological lesions are also more common. Together, these findings point to a more direct BDV-induced effect in the development of clinical signs in feline BD than has been considered previously.

Future Perspectives

Borna disease virus is an intriguing infectious agent, and after a century of research concerning different aspects of this pathogen and the diseases it causes, many questions are still to be answered. From a clinical point of view, better and more secure diagnostic methods are needed, as well as increased knowledge of how to interpret diagnostic results, and what to do when a diagnosis is made. Further studies to enlighten the infection biology, as well as the molecular epidemiology, of BDV are also needed.

Are the typical clinical signs enough to determine whether a particular cat suffers from feline BD? Or is a serological test needed for confirmation? If the test is negative, does this mean that the cat does not have the disease? If a cat showing atypical clinical signs carries antibodies, how likely is the clinical diagnosis to be feline BD? Even though these questions have been partially answered in this thesis, further studies are needed, such as sero-epidemiological studies of cats from different geographical regions of Sweden, and with different housing, as well as more concentrated studies of cats showing different neurological signs. Other species, like horses and dogs, should also be included, since BDV-infection is known in these species in Sweden as well (unpublished data; Berg *et al.,* 1999a; Berg *et al.,* 1998b).

Even if a confident diagnosis can be made, is that of importance if the outcome is still euthanasia, based on bad prognosis and animal welfare concerns? Other diseases could be similar to feline BD clinically, and thus a confirmation of the diagnosis is important. Once a confident diagnostic test is available, evaluations of different treatments would be necessary. In horses with BD, antiviral therapy using amantadine is reported to be favorable in several cases (Dieckhöfer, 2008; Dieckhöfer *et al.*, 2004). This treatment has been used in cats as well, and in at least one case the cat got better, although side effects were present (Stina Viktorsson, personal communication). This

cat was determined as positive for BDV-RNA in blood by rRT-PCR (unpublished data), although unfortunately it was not possible to follow up this case after treatment. Another therapy used in cats is corticosteroid treatment, which seems to be beneficial, especially if it is introduced at an early stage of the disease (Berg, 1999), although the effect is often seen only temporarily (Karin Hultin Jäderlund, personal communication). However, even if immunosuppressive treatments, such as corticosteroid therapy, reduce the inflammatory response to BDV-infection, and hence immune-mediated clinical signs, the immunosuppression could lead to higher replication of virus. A thorough evaluation of different therapies of feline BD would be profitable for investigating whether this disease could be treated.

Feline BD has mostly been reported from Sweden, though there are studies from other parts of the world as well, indicating a wide geographical distribution of this disease (Kamhieh & Flower, 2006). Recently, a similar neurological disorder of unknown etiology was reported from the United Kingdom (De Risio *et al.*, 2008), where BDV-infections in cats and a horse have been demonstrated (Priestnall *et al.*, 2011; Reeves *et al.*, 1998). In these so-called "robotic cats", clinical signs are similar to those presented in Paper II. Some of these cats were investigated for the presence of BDV-infection markers, although no such markers were detected. It would be worth increasing the search for BDV-infection markers in these cats, possibly including detection systems for more divergent strains like ABV. Serological evidence for BDV-infection in large felids (lions) was recently reported from Poland (Czujkowska *et al.*, 2011). However, these findings need to be further confirmed by other methods. Thus, epidemiological studies, including molecular epidemiology, for neurological disorders similar to feline BD are needed.

Different natural reservoirs for BDV have been discussed, though there are arguments for direct spread of BD in horses as well, because of the high sero-prevalence. The spread of BDV to different domesticated species could be variable, depending on housing and feeding preferences. Hypothetically, direct spread could be of more importance in herbivores, such as horses and sheep, whereas carnivores, such as dogs and cats, need to feed on infected prey to become infected. If this hypothesis could be confirmed, it would be an explanation for why BDV-infection in cats and dogs is not as frequently reported as in herbivores. Cats and dogs could in this case be regarded as accidental hosts, when preying on infected herbivores like rodents.

In other members of the order of *Mononegavirales*, the Marburg and Ebola viruses, it has been speculated that these viruses are originally arthropod or

plant viruses transmitted to animals when they either feed on the plants or are bitten by the insects (Monath, 1999). Interestingly, the gene of BDV L has the most genetic similarity with a plant virus belonging to the *Nucleorhabdoviruses*, the only genus within the same order (besides BDV) to replicate inside the nucleus of infected cells (Pringle, 2005). Thus, although highly speculative, plant or insect viruses could be the ancient origin of BDV, and wild herbivores, like rodents, could have acted as mixing vessels. This hypothesis would be interesting to follow up, to find out whether there is a genetic relationship between BDV and RNA-viruses of plants and/or arthropods, and also to follow up serological evidence of BDV in wild rodents (Kinnunen *et al.*, 2007) by molecular characterization of such viruses. For such studies, viral metagenomics could be useful, where the global viral flora can be detected by sequence-independent methodology (Blomström, 2011), in order to find new, divergent BDV strains or BDV-like agents. In this context, the recent findings of the genetically more divergent ABV are also interesting.

There is limited or rather poor information available concerning the molecular epidemiology of feline strains of BDV, and there is a great need for characterizing these strains further for greater knowledge. For example, are these strains more similar to ABV, than previously considered? Because of the low viral load in cats, these kinds of studies are troublesome. During the work for this thesis, we have tried to isolate virus from naturally infected cats in different cell lines, though so far these efforts have failed. At least one BDV-strain isolated from cats would be helpful for further characterizing feline BDV by full-genome sequencing. Also, the viral metagenomic approach could be useful, to detect novel BDV strains, or other pathogens, coinciding in feline BD. Co-infections could be one explanation for why not all cats carrying BDV-specific antibodies develop clinical disease. Host factors, like different genetic background, could be another explanation for this observation.

The pathogenesis of BD in different species still needs further elucidation. The cellular receptor for BDV when entering cells in the olfactory epithelium is still unknown, and inside the CNS the kainate receptor has been proposed to be involved in viral entry (Gosztonyi, 2008). By what mechanisms the RNP is transported from the first site of entry to the CNS is also unknown, although most likely the macromolecular transport systems within neural cells are used. The direct viral-induced effects contributing to neurological signs are also interesting subjects for further studies. In all of these investigations, the application of *in situ* PLA could be a practical tool to study BDV-host protein-protein interactions in

brain tissues of experimentally and naturally infected animals. Further cytokine mRNA expression analyses in cats with feline BD, accompanied by comparative studies of BDV infection in other species, could also contribute to a better understanding of the host immune responses, as well as viral evasion mechanisms, in this intriguing disease.

Populärvetenskaplig sammanfattning

Vingelsjuka hos katt är en neurologisk sjukdom som karakteriseras av vinglig gång och beteendeförändringar. Denna sjukdom orsakas av ett virus, bornavirus, som ger en kvarstående (persistent) infektion av celler i centrala nervsystemet. Bornavirus-infektioner har varit kända sedan början av 1900-talet hos häst och får i Centraleuropa, men det var först i mitten av 1990-talet som det upptäcktes att vingelsjuka hos katt, beskriven första gången i början av 1970-talet, orsakas av bornavirus. Däremot har det hittills varit svårt att ställa diagnosen vingelsjuka eller felin bornasjuka hos levande katter.

Målet med denna avhandling var att utveckla nya metoder för att påvisa virus, i syfte att förbättra den nuvarande diagnostiken. Ett annat mål var att öka kunskapen om interaktioner mellan virus och värd, för att bättre förstå hur sjukdom hos djur infekterade med bornavirus uppkommer. Det senare gjordes med molekylära tekniker, där virusproteinerna studerades, särskilt med avseende på hur de binder till värdens proteiner och därmed påverkar deras normala funktion.

I den första studien utvecklades en molekylärdiagnostisk metod för påvisande av virus-nukleinsyra. Denna metod är mycket känslig, eftersom endast 10 kopior av virusets arvsmassa kunde detekteras. Dessutom är metoden bred i sin detektionsförmåga, eftersom den kunde påvisa olika referensstammar av bornavirus, liksom virus från katter med vingelsjuka. I nästa studie användes denna metod, men även en serologisk metod för påvisande av antikroppsförekomst i blodserum, för att utvärdera vilken eller vilka metoder som är bra för att bekräfta bornavirusinfektion hos katter med vingelsjuka. Det visade sig att antikroppar i serum är en mycket bra och säker hjälp vid diagnostik av vingelsjuka, i och med att 81 % av katterna med kliniska symtom på vingelsjuka hade antikroppar, jämfört med endast 16 % hos en referensgrupp av katter utan dessa symtom.

I den tredje studien användes en ny metod (så kallad närhetsligering) för att se interaktioner mellan virusets protein och värdorganismens protein. Sådana interaktioner kunde ses i experimentellt infekterade celler, men även för första gången i en experimentellt infekterad råtta och i en häst med bornasjuka. Sannolikt orsakar dessa interaktioner störningar hos värddjurets normala funktioner, vilket underlättar för viruset att etablera en långvarig infektion och gömma sig för immunsvaret.

En del i detta immunsvar är signalmolekyler som kallas interferoner (IFN), av vilka IFN-γ är särskilt viktig för att minska mängden virus i hjärnan. I den sista studien studerades uttrycket av IFN-γ hos katter med vingelsjuka. Det visade sig att detta uttryck är kraftigt vid vingelsjuka och kraftigast hos katter med milda kliniska symtom. Denna grupp av katter har också de allvarligaste sjukliga förändringarna i hjärnan. Sammantaget pekar det mot att bornavirus kan ha en mer direkt påverkan i utvecklingen av de symtom som ses vid vingelsjuka än vad man tidigare trott.

Fler framtida studier behövs för att ytterligare klargöra sjukdoms-mekanismerna bakom denna mystiska sjukdom, samt för att få mer kunskap om hur sjukdomen sprids och om den kan behandlas.

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