OPEN ACCESS
British Journal of Virology

Research Article

Visualization of Borna Disease Virus Protein Interactions with Host Proteins using *in situ* Proximity Ligation Assay

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Abstract | Borna disease virus type 1 (BDV) comprises highly conserved neurotropic non-segmented negative strand RNA-virus variants causing neurological and behavioral disorders in a wide range of mammalian animals, possibly including humans. Viral persistence in the brain has been frequently observed, however, the exact mechanisms behind BDV's ability to establish persistence despite a prominent immune response are not known. Here we have used *in situ* proximity ligation assay (*in situ* PLA), a selective tool for studying virus-host protein-protein interactions. BDV P (phosphoprotein) and N (nucleoprotein) have previously been reported to interact with several host proteins, thereby interfering with various signaling pathways. In this study, we focused on some of these interactions (BDV P-HMGB1, BDV N/P-Cdc2). First, we used rat glioma cell cultures persistently infected with a laboratory strain of BDV (C6BV) to establish the assay. Next, *in situ* PLA was applied to detect BDV P in brain tissues of infected animals. Finally, protein-protein interactions were visualized in both C6BV and brain tissues of experimentally as well as naturally infected animals (rat and horse, respectively). BDV proteins and their interactions with host proteins could be shown in cell cultures (HMGB1, Cdc2) and in brain tissues of rat (HMGB1, Cdc2) and horse (Cdc2 only) infected with BDV. In this study, we have for the first time directly visualized protein-protein interactions between BDV and its host, and thereby confirmed previous data to demonstrate findings in cell cultures to be applicable also in experimentally and naturally infected animals.

Editor | Muhammad Munir, The Pirbright Institute, UK.
Received | February 11, 2016; **Accepted** | March 06, 2016; **Published** | March 09, 2016
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DOI | http://dx.doi.org/10.17582/journal.bjv/2016.3.1.11.23
Citation | Wensman, J. J., K. Leuchowius, J. Yan, A. Berg, L. Bode, H. Ludwig, S. Belák, U. Landegren, O. Söderberg and M. Berg. 2016. Visualization of Borna disease virus protein interactions with host proteins using in situ proximity ligation assay. *British Journal of Virology,* 3(1): 11-23.
Keywords | *Bornavirus*, Virus-host interactions, Viral persistence, Cdc2, HMGB1

Introduction

The family *Bornaviridae* of the order *Mononegavirales* has currently been taxonomically reorganized to allow for classifying numerous new viruses discovered in various bird species, which are genetically different from mammalian bornavirus (Kuhn et al., 2015). Psittaciform and passeriform bornavi-

ruses cause proventricular dilatation disease (PDD) in psittacine and non-psittacine birds (Kistler et al., 2008; Weissenböck et al., 2009). This work, however, focuses on classical Borna disease virus type 1 (BDV; genus *Bornavirus*, species *Mammalian 1 bornavirus*). All variants/strains are characterized by highly conserved genomes, persistently infecting neurons and glia cells mainly in the central nervous system (CNS). They have been shown to cause neurological diseases in a wide range of mammals, including Borna disease in horses and sheep (Lipkin and Briese, 2006; Ludwig et al., 1988), as well as staggering disease in cats (Wensman et al., 2014). Human isolates have been reported from Germany (Bode et al. 1996) and Japan (Nakamura et al. 2000). Endogenous bornavirus N-like elements (EBLNs) resembling the N-gene of BDV were found in the genomes of humans, primates, and other mammal species (Belyi et al., 2010; Horie et al., 2010), suggesting that bornaviruses are evolutionarily old viruses, co-existing with humans and their predecessors for a long time. Some of the EBLNs found in the human genome are conserved as protein-coding genes (Belyi et al., 2010; Horie et al., 2010). These findings could point to a relationship of BDV infections and psychiatric diseases (Feschotte, 2010), as has been previously suggested (Bode and Ludwig, 2003). Moreover, variegated squirrel 1 bornavirus (VSBV-1) was recently proposed to underlie three cases of fatal viral encephalitis in human (Hoffmann et al., 2015).

The BDV genome (ca. 8.9 kb) constitutes a non-segmented, single-stranded RNA of negative polarity with six open reading frames (ORFs) encoding the nucleoprotein (N), phosphoprotein (P), a small non-structural protein (X), matrix protein (M), glycoprotein (G) and the RNA-dependent RNA-polymerase (L), a genome organization similar to that of other *Mononegavirales* (Lipkin and Briese, 2006). Nuclear replication, however, characterizes a unique feature of *Bornaviridae* (Cubitt and Torre, 1994), involving intense shuttling of proteins between nuclear and cytoplasmic compartments. Despite recent progress, the pathogenesis of bornavirus infections is poorly understood. Immune-mediated pathological processes are considered to lead to the development of clinical signs in infected animals (Stitz et al., 1995). Even direct effects of interactions between BDV P and host may also induce clinical signs (Kamitani et al., 2003). *In vitro*, several interactions are known to take place between BDV proteins and those of its host. For example, BDV N interacts with the Cdc2-Cyclin B1 complex (Planz et al., 2003), possibly interfering with the cell cycle. BDV P interacts with TBK1 (Unterstab et al., 2005), a kinase activating IRF-3 and -7 followed by type I IFN expression. Furthermore, BDV P has also been shown to interact with the gamma-aminobutyric acid receptor-associated protein (GABARAP) (Peng et al., 2008), presumably affecting GABA neurotransmission, as well as with the nuclear protein high-mobility group box-1 (HMGB1) (Kamitani et al., 2001). HMGB1 is a multifunctional protein involved in transcriptional regulation (Ueda and Yoshida 2010), neurite outgrowth (Rauvala et al., 2000), cell migration (Rauvala and Rouhiainen 2010), and DNA repair (Liu et al., 2010), but is also recognized as an alarmin, released upon tissue damage and infection to induce a pro-inflammatory response (Yang et al., 2010). Thus, interactions between BDV P and HMGB1 could be a means for BDV to evade the host immune response by reducing release of HMGB1 and its induction of inflammation.

Cdc2, also known as cyclin-dependent kinase 1 (Cdk1), forms a complex with Cyclin B1. When Cyclin B1 is phosphorylated, the Cdc2-Cyclin B1 complex is translocated into the nucleus. This translocation is needed for cells to enter the M phase. If activation of the Cdc2-Cyclin B1 complex fails, cells can be driven into apoptosis. BDV N has previously been shown to interact with both phosphorylated and non-phosphorylated Cdc2, as well as with Cyclin B1 (Planz et al., 2003), whereas BDV P was shown to interact only with the non-phosphorylated form of Cdc2. BDV N reduced the proliferation rate of transfected rat fibroblast cells, while this was not seen in BDV P transfected cells. A reduced proliferation rate and enhanced apoptosis were also seen in BDV-infected oligodendroglial (OL) cells, but induced by a natural human strain, whereas a non-natural laboratory strain (strain V) displayed the opposite effects (Li et al., 2013). Taken together, these data indicate that BDV strains of different origin are able to differently interfere with cell proliferation. One mechanism in focus here is through interaction between its nucleoprotein and the Cdc2-Cyclin B1 complex, thus paving the way for establishing a persistent infection.

Most of the host-BDV protein-protein interactions in the literature were demonstrated in the course of *in vitro* studies and in infected cell cultures. Interaction between HMGB1 and BDV P has been investigated

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Table 1: *Antibodies used for IHC and in situ PLA*

Antibody target	Species of origin and clonality	Dilution in cell cultures	Dilution in tissues	Reference or supplier
BDV P	Rabbit, polyclonal	1:50 000	1:10 000	Johansson et al., 2002
BDV P	Mouse, monoclonal (Kfu2)	1:50	1:50	Ludwig et al., 1993; Bode, 2008
BDV N	Rabbit, polyclonal	1:25 000	1:5 000	Johansson et al., 2002
BDV N	Mouse, monoclonal (W1)	1:50	1:50	Ludwig et al., 1993; Bode, 2008
Human HMGB1	Rabbit, polyclonal	1:16 000	1:1 000	ab18256, Abcam, Cambridge, UK
Xenopus Cdc2	Mouse, monoclonal (A17)	1:500	1:500	ab18, Abcam

Antibodies were used as indicated for IHC and in situ PLA in cell cultures and tissues. In cell cultures, the antibodies were initially evaluated and titrated using standard immunofluorescence assay

using far-Western blotting, BDV P affinity chromatography, mammalian two-hybrid analysis, and co-immunoprecipitation (Kamitani et al., 2001). In addition, studies have focused on biological effects of this interaction on neurite outgrowth and p53-mediated transcription (Kamitani et al., 2001; Zhang et al., 2003). BDV N and P interactions with Cdc2-Cyclin B1 were demonstrated by GST-pulldown and Western blotting (Planz et al., 2003). As mentioned above, there have also been studies of possible biological effects of the latter interactions. However, none of these interactions have been demonstrated or investigated in experimentally or naturally infected animals. Thus, the impact of these interactions in infected animals still needs to be elucidated.

Here, we have addressed this question by using *in situ* proximity ligation assay (*in situ* PLA) to visualize BDV-host interactions in cell cultures and in brain tissues of experimentally and naturally infected animals using a non-natural commonly applied laboratory strain. We expect that *in situ* PLA will create new opportunities for studies of pathogenesis and virus-host interactions in tissues of naturally infected animals. Accordingly, this approach may contribute to increased understanding of the pathogenesis of infectious diseases.

Materials and Methods

Virus and Cells

Uninfected rat C6 glioma cells or cells persistently infected with BDV strain He/80 (C6BV; Cubitt et al., 1994) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 5% fetal bovine serum (FBS), 1% L-glutamine 200 mM and 1% penicillin-streptomycin solution at 37°C and 5% CO_2. For *in situ* PLA, cells were cultured in LabTek® II Chamber slides (Nunc, Rochester, NY). Cells were washed three times in PBS for 5 minutes, and then fixed in acetone for 10 minutes.

Animal tissues

Formalin-fixed paraffin-embedded (FFPE) brain tissue was obtained from a Wistar rat, intra-cerebrally inoculated with laboratory BDV strain V at three months of age and sacrificed at approximately 30 days post infection. After euthanization, rat tissues were fixed by perfusion to allow good conservation of viral and other epitopes. Further, FFPE brain tissue from a naturally infected horse showing classical signs of BD (loss of weight, ataxia, circling movement, depression) was used. The horse was about 12 years old when euthanized. FFPE brain tissues from the infected rat and horse were kindly provided by Professor Georg Gosztonyi, FU Berlin, Berlin, Germany. Brain tissues from an uninfected rat (kindly provided by Dr Jonas Tallkvist, SLU, Uppsala, Sweden) and uninfected horses were used as negative controls. Sections were cut on SuperFrost Plus slides and kept at room temperature until de-paraffinization. After de-paraffinization, heat-induced epitope retrieval (HIER) was performed in citrate buffer at pH 6.0 (Dako, Glostrup, Denmark).

Antibodies

Information about antibodies is given in Table 1. Generation and characterization of specificity of the rabbit polyclonal anti-BDV P and anti-BDV N antibodies, as well as the mouse monoclonal anti-BDV P and anti-BDV N antibodies, has been described earlier (Bode, 2008; Johansson et al., 2002; Ludwig et al., 1993).

Immunohistochemistry

HIER-treated rat brain tissues were subjected to immunohistochemical (IHC) staining for BDV P with Envision+ kit (Dako), according to the manufacturer's

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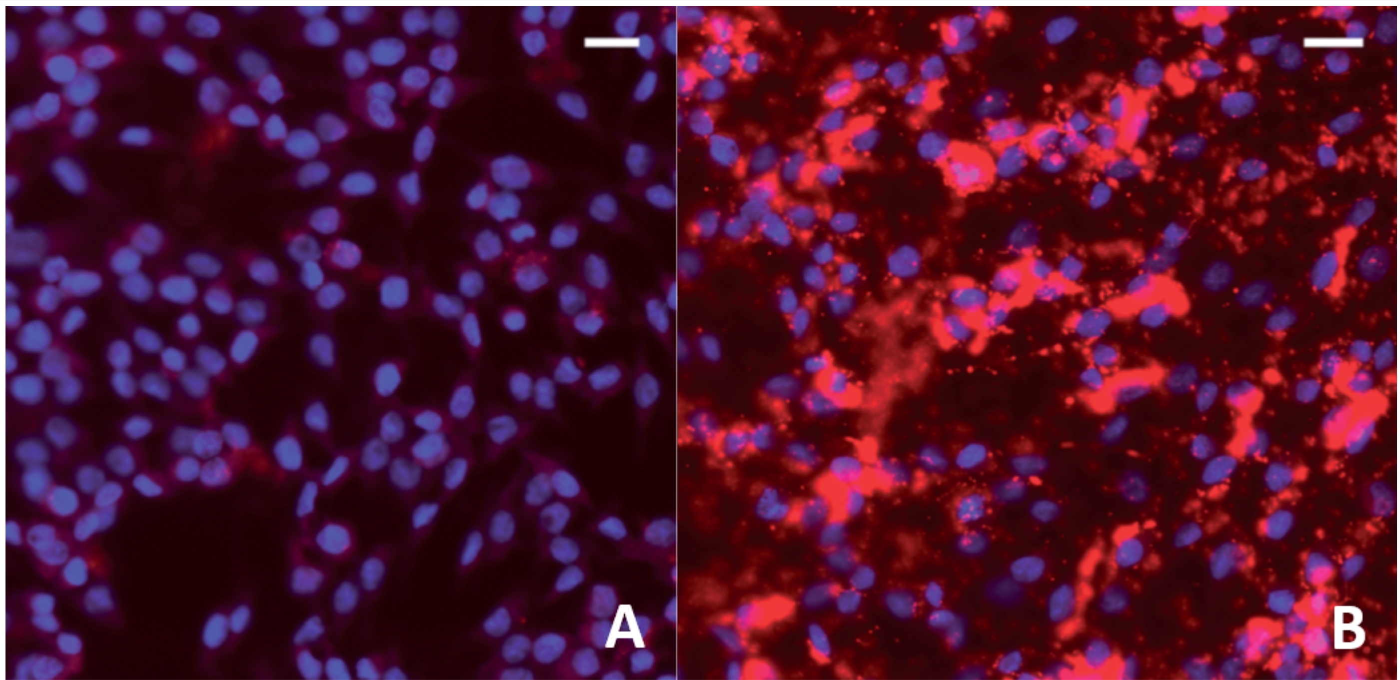

Figure 1: *Detection of BDV P using in situ PLA in cell cultures*
In situ PLA using a rabbit polyclonal anti-BDV P antibody and a monoclonal anti-BDV P antibody (dilution 1:100) was performed in **A)** *C6 and* **B)** *C6BV cell cultures. The in situ PLA signals (BDV P) are seen as red dots, nuclei are blue. Notice the oversaturated staining of BDV P, especially in nuclei of C6BV, resulting from several in situ PLA signals coalescing. Magnifications: lens x20.*

instructions. IHC staining was also used to confirm the reactivity of antibodies against host proteins, using the Vector ABC Elite kit (Vector lab, Burlingame, CA). Mayer's hematoxylin (Histolab, Gothenburg, Sweden) was used to stain cell nuclei.

In situ PLA

In situ PLA was performed using the Duolink kit (Olink Bioscience AB, Uppsala, Sweden). Briefly, fixed cell cultures or HIER-treated tissues were immersed in a blocking solution (TBS with 5% goat serum) for 60 minutes at room temperature to reduce unspecific binding of the antibodies. Primary antibodies were then diluted in blocking solution and applied to cell cultures or tissues overnight at 4°C. After washing with TBS, PLA probes (oligonucleotide-conjugated anti-rabbit PLUS and anti-mouse MINUS antibodies for cell cultures, anti-mouse PLUS and anti-rabbit MINUS antibodies for tissues) were diluted 1:5 in blocking solution and applied to cell cultures or tissues for 120 minutes at 37°C. Detection of PLA probes was then performed with the Duolink detection kit 563 (cell cultures) or 613 (tissues) according to the manufacturer's instructions.

To reduce problems with background autofluorescence in the FFPE tissues, as well as to improve morphological analysis, fluorescence *in situ* PLA signals were replaced by chromogenic signals for brightfield microscopy through the use of DuoCISH kit (Dako). Cell nuclei were stained with Mayer's hematoxylin (Histolab).

Fluorescence microscopy was performed using a Zeiss AxioPlan II epifluorescence microscope with a Zeiss HRm CCD camera. Brightfield imaging was performed using a Nikon Eclipse E600 with a Deltapix 200 camera. Images of *in situ* PLA stained cell cultures were analyzed with the freeware software Blobfinder (Allalou and Wahlby, 2009) or with Duolink ImageTool (Olink Bioscience) to quantify numbers of *in situ* PLA signals per cell.

Results and Discussion

Detection of BDV P in persistently infected cell cultures and brain tissues from infected animals using *in situ* PLA

To evaluate and validate the *in situ* PLA technique for investigating BDV-infected cell cultures, one monoclonal and one polyclonal antibody directed against BDV P were applied to C6 cells, either persistently infected with C6BV or uninfected. As shown in Figure 1, there was a strong, specific fluorescent signal in C6BV infected cells as compared to uninfected cells.

Next, brain tissues from an experimentally infected rat and a negative control rat were investigated. First

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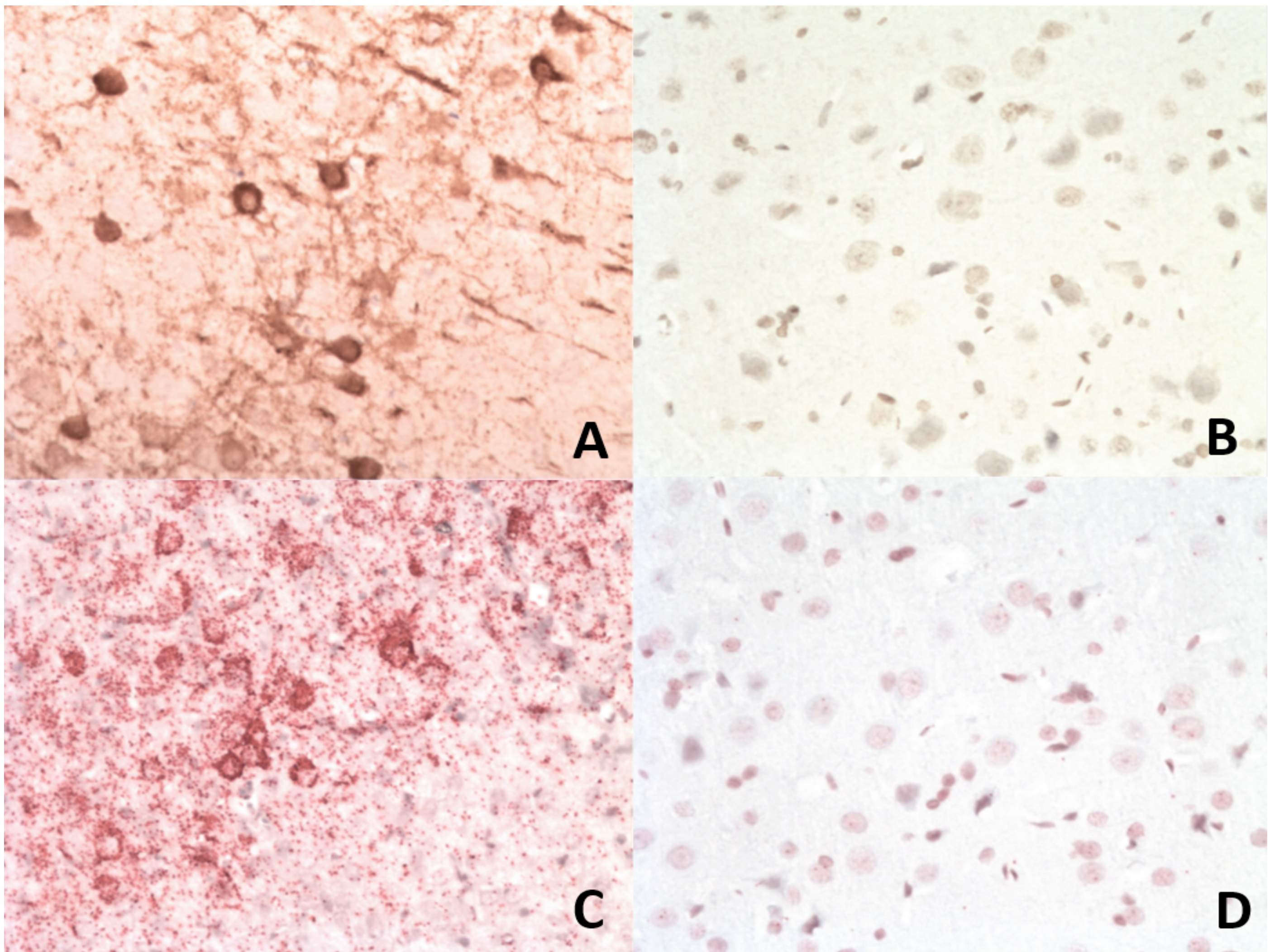

Figure 2: *BDV P IHC and in situ PLA on brain tissues from an experimentally infected rat and an uninfected control* **A)** *IHC of BDV P on brain tissue from an experimentally infected rat using a rabbit polyclonal anti-BDV P antibody. The brown staining is BDV P.* **B)** *IHC of BDV P on brain tissue from an uninfected control rat.* **C)** *In situ PLA of BDV P on brain tissue from the same rat as in A, using rabbit polyclonal and mouse monoclonal anti-BDV P antibodies. The red staining is BDV P.* **D)** *In situ PLA of BDV P on brain tissue from the same uninfected control rat as in B. Magnifications: lens x20.*

a polyclonal anti-BDV P antibody was used for IHC staining. As shown in Figure 2A and B, neurons were intensely stained in the experimentally infected rat, while no specific staining was observed in the negative control rat. However, in the cerebellum of the uninfected rat, there was a slight diffuse unspecific background staining in the granular cell layer, probably caused by endogenous peroxidase (not shown). *In situ* PLA, using the same combination of BDV P antibodies as had been applied to the cell cultures, gave strongly positive signals in the form of red dots labeling the neurons in the BDV-infected rat brain, while no positive signals were present in the uninfected rat brain (Figure 2C and D). Similar diffuse unspecific background staining in the cerebellum as seen with IHC was present also using *in situ* PLA (not shown).

To further evaluate *in situ* PLA for detection of viral proteins in comparison with IHC, we applied these two techniques to brain tissue from a horse with confirmed BD. As seen in Figure 3A and B, there was a strong positive signal for BDV P in neurons of this horse using IHC, but not in brain tissue from the negative control horse. A similar distribution pattern was seen using *in situ* PLA (Figure 3C and D).

Although the overall distribution pattern of positively stained neurons was approximately similar using IHC and *in situ* PLA, fewer BDV-positive cells were observed when *in situ* PLA was applied. This difference may be explained by the requirement for dual recognition in the *in situ* PLA; i.e., both polyclonal and monoclonal anti-BDV P antibodies need to bind their target epitopes to give rise to a signal. In fact, epitope mapping has shown that the monoclonal anti-BDV P antibody binds specifically to phosphorylated BDV

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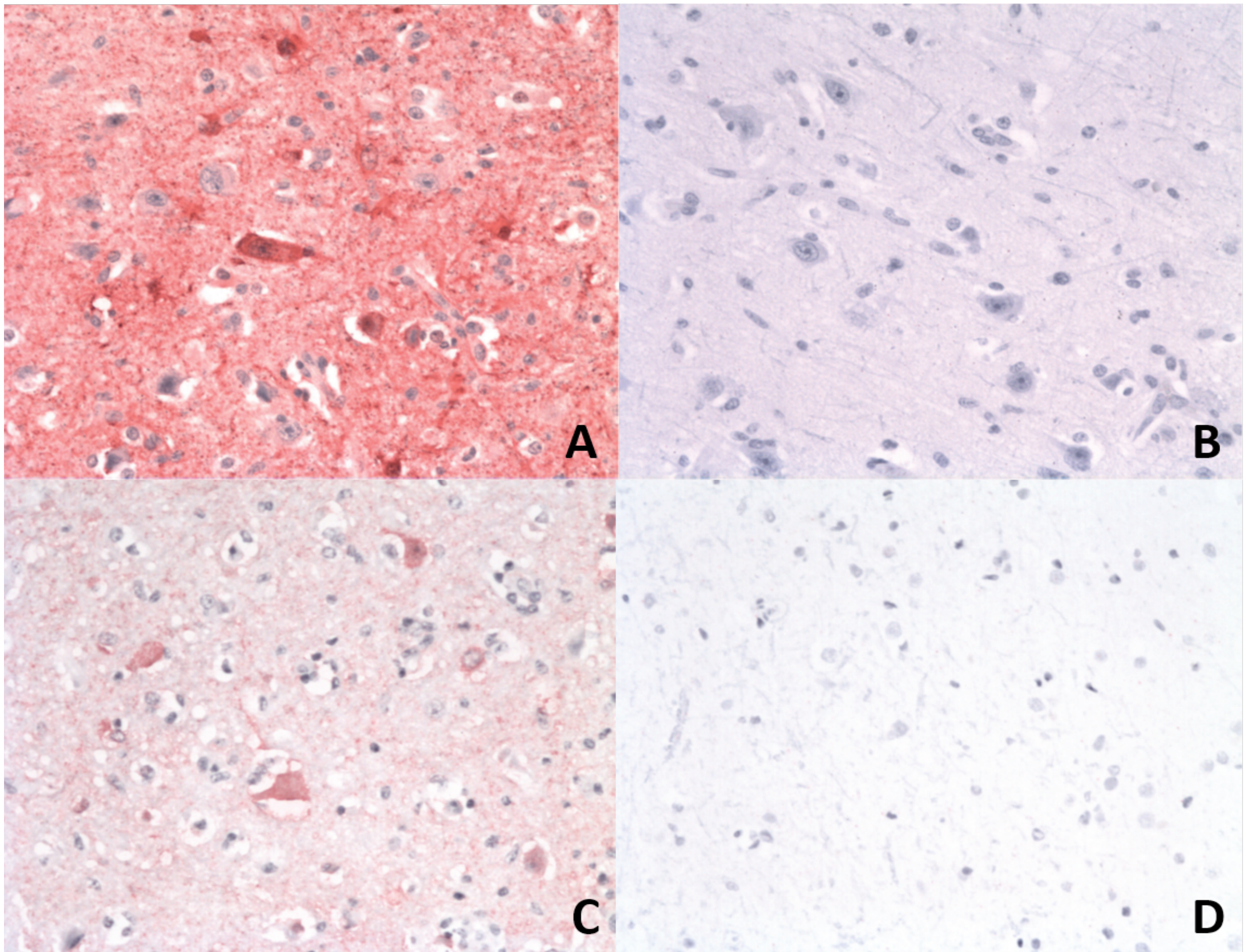

Figure 3: *BDV P IHC and in situ PLA on brain tissues from a naturally infected horse and uninfected controls*
A) *BDV P IHC on brain tissue from a naturally infected horse using a rabbit polyclonal anti-BDV P antibody. The red staining is BDV P.* **B)** *IHC of BDV P on brain tissue from an uninfected control horse.* **C)** *In situ PLA of BDV P on brain tissue from the same horse as in A, using rabbit polyclonal and mouse monoclonal anti-BDV P antibodies. The red staining is BDV P.* **D)** *In situ PLA of BDV P on brain tissue from an uninfected control horse. Magnifications: lens x20.*

P, which was also confirmed by determining *in vitro* phosphorylated recombinant BDV P in a BDV antigen EIA (Bode, 2008). The *in situ* PLA approach can therefore provide a more selective detection.

BDV P is phosphorylated mainly at serine residues 26 and 28 by PKCε, but also to a lesser extent at serine residues 70 and 86 by casein kinase II (CKII) (Schwemmle et al., 1997). Phosphorylated BDV P down-regulates viral polymerase activity, in contrast to other mononegaviruses (Schmid et al., 2007). However, PKC phosphorylation of BDV P is needed for efficient viral spreading, indicating other important functions of phosphorylated BDV P (Schmid et al., 2010). Furthermore, BDV P seems to competitively interfere with normal PKC substrates, affecting neuronal plasticity (Prat et al., 2009). However, further investigations are needed to demonstrate the importance of phosphorylated BDV P, for example for virus spread, and local distribution in different stages of disease in infected animals.

Visualization of virus-host protein-protein interactions in cell cultures

The possibility of being able to study protein-protein interactions in cell cultures and tissues represents an important advantage of *in situ* PLA over other *in situ* analysis techniques. We chose to target previously published interactions between BDV proteins and host proteins (BDV P-HMGB1 and BDV N/P-Cdc2) to investigate this opportunity. C6BV and C6 cell cultures were used to study protein-protein interactions by *in situ* PLA. A clear difference in the number of signals was seen between C6BV and C6 c

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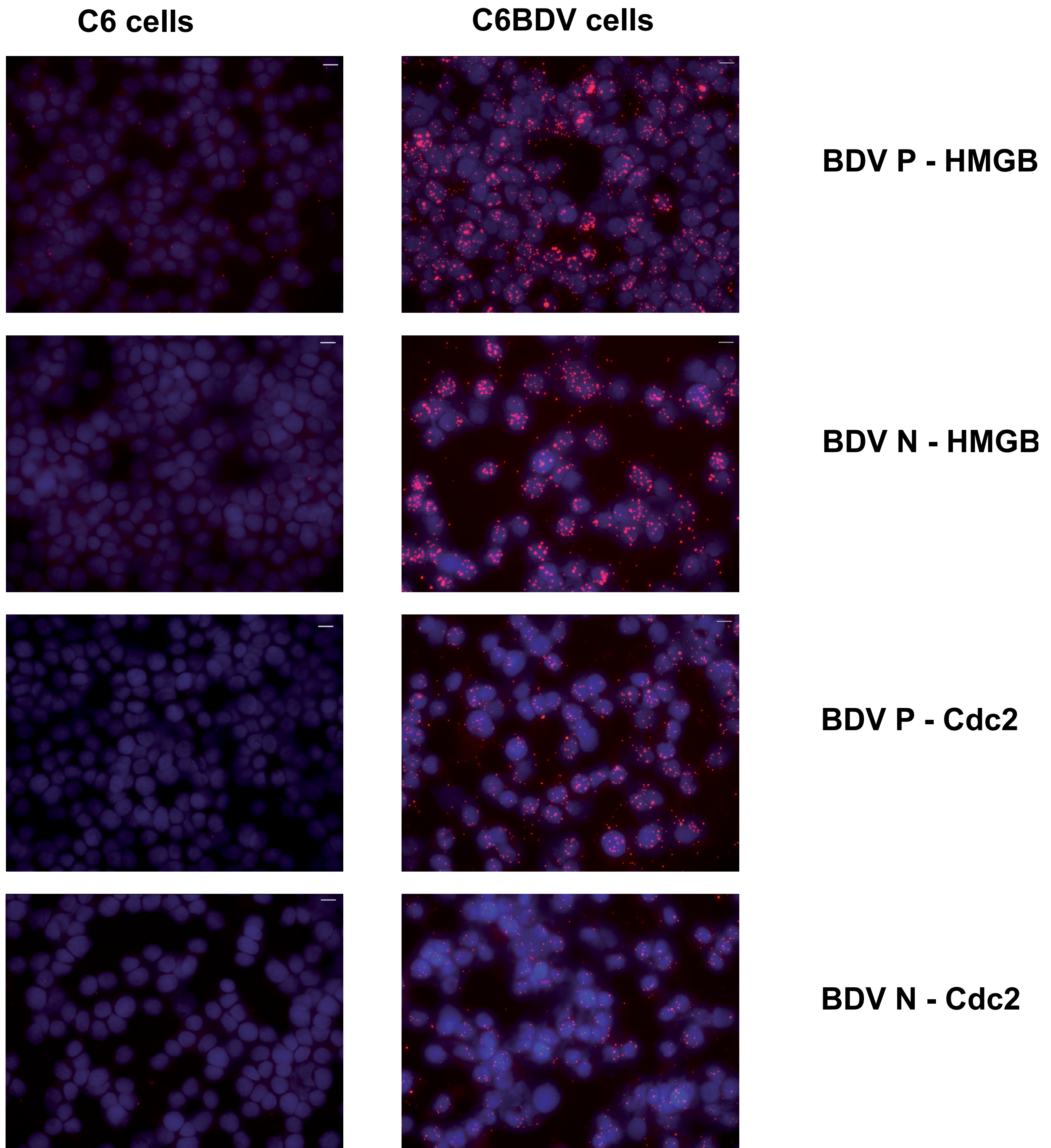

Figure 4: *Visualization of host-virus protein-protein interactions in persistently infected cell cultures*
In situ PLA for the indicated protein-protein interactions was performed in persistently BDV-infected cell cultures (C6BV), using uninfected C6 cells as negative control. Intense positive signals could be seen in the C6BV cells, especially in the nuclei, compared to the C6 cells. The number of signals per cell was quantified using Duolink ImageTool. The results of the quantifications are shown in Figure 5.

ells in all tested interactions (Figure 4). This allowed us to visualize for the first time *in situ* BDV-host protein-protein interactions previously demonstrated only in cell free contexts.

Signals were quantified using the Duolink Image Tool (Figure 5). For all protein-protein interactions, there seem to reside more signals inside the nuclei than in the cytoplasms. These results also indicate that HMGB1 interacts not only with BDV P but also with BDV N. However, BDV P and BDV N are known to interact with each other (Berg et al., 1998;

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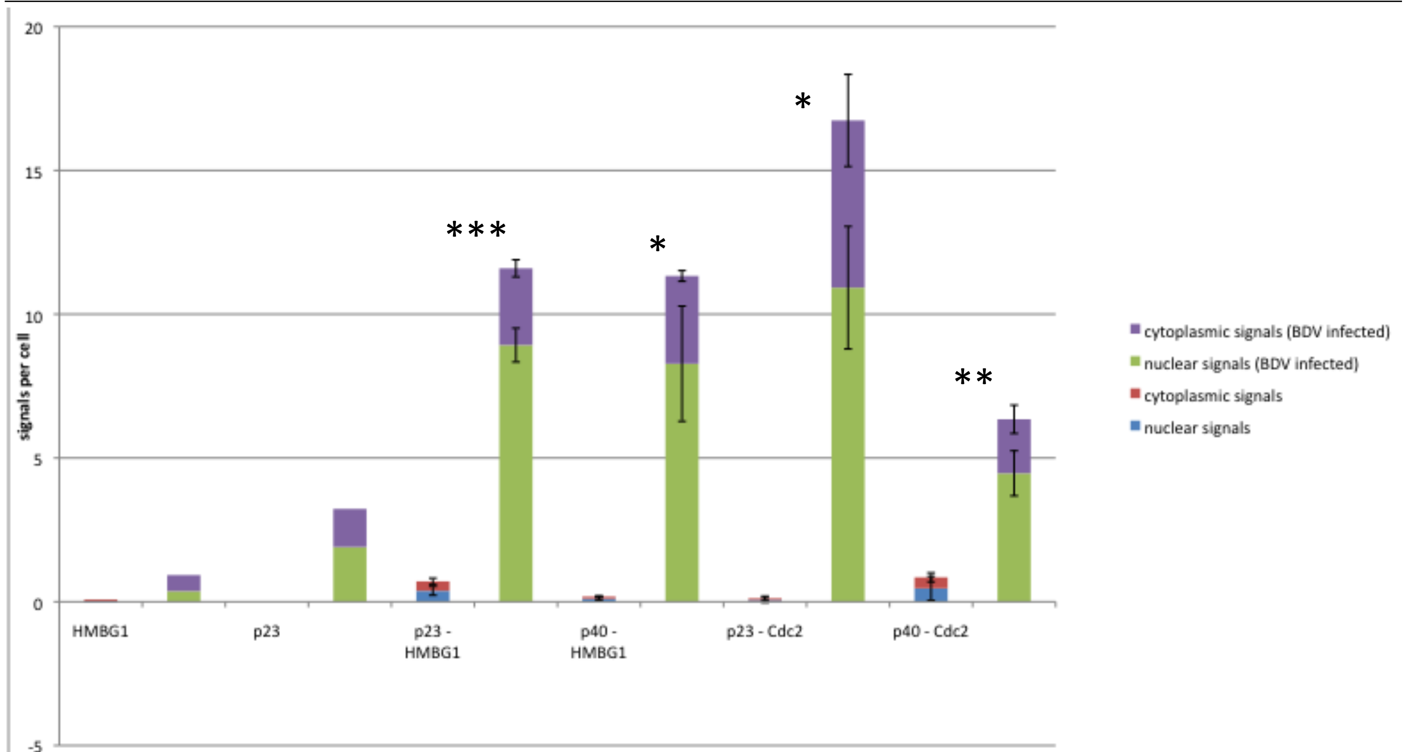

Figure 5: *Quantification of host-virus protein-protein interactions in persistently infected cell cultures*
*The images of the same experiments as shown in Figure 4 were analyzed using Duolink ImageTool (Olink Bioscience). In total, three images were analyzed for each interaction. The number of signals per cell is shown as the cumulative nuclear (green for C6BV cells, blue for C6 cells) and cytoplasmic (purple for C6BV cells, red for C6 cells) signals of the indicated interaction. To the left, two technical controls are shown, where only one of the primary antibodies (as indicated) was used. All other steps were the same as for all other interactions. Statistical analysis (Student's t-test) was performed using Microsoft Excel, where * indicates p≤0.05, ** p≤0.01 and *** p≤0.001. p23 = BDV P. p40 = BDV N.*

Bode, 2008). Although data suggest that BDV N and HMGB1 are components of a protein complex, it does not conclusively prove that these proteins are physically interacting. Based on previous data using the mammalian two-hybrid system (Kamitani et al., 2001), it seems likely that the signals seen here using antibodies towards BDV N and HMGB1 arise from indirect interaction via BDV P and intermediary proteins.

Visualization of virus-host protein-protein interactions in brain tissues of experimentally and naturally infected animals

There are no previous data on BDV-host protein-pro tein interactions in experimentally or naturally infected animals. Therefore, the importance of these interactions in the pathogenesis of BDV infection in animals is not known. In this study, we used *in situ* PLA to visualize virus-host protein interactions in brain tissues. First, reactivity of antibodies directed against the host proteins was confirmed in brain tissues of an uninfected rat and horse by IHC (data not shown). Next, we performed *in situ* PLA to visualize interactions previously detected in cell cultures. We compared brain tissue from the experimentally infected rat and naturally infected horse with uninfected controls. As a technical control we also left out one of the primary antibodies used in the *in situ* PLA. Since both primary antibodies need to bind in close proximity to obtain a positive reaction, a single primary antibody should not give rise to signals. Thereby, any signals in this control would come from unspecific binding of secondary antibodies or detection oligonucleotides. As expected, few if any signals were found in this technical control (Figure 6E).

In the experimentally infected rat, interactions between BDV P and HMGB1, shown as distinct red dots, could be detected in scattered neurons (Figure 6A). No positive signals were detected in the uninfected control rat (Figure 6B). In contrast, interactions between BDV P and HMGB1 could not be demonstrated in the naturally infected BD horse.

HMGB1 is recognized as an alarmin, released upon danger signals such as infection and/or tissue damage (Yang et al., 2010). Released proteins act as ligands to Toll-like receptor 4 (TLR4) and receptor for advanced glycation end-products (RAGE) (Yang et al., 2010). Binding of HMGB1 to RAGE leads to

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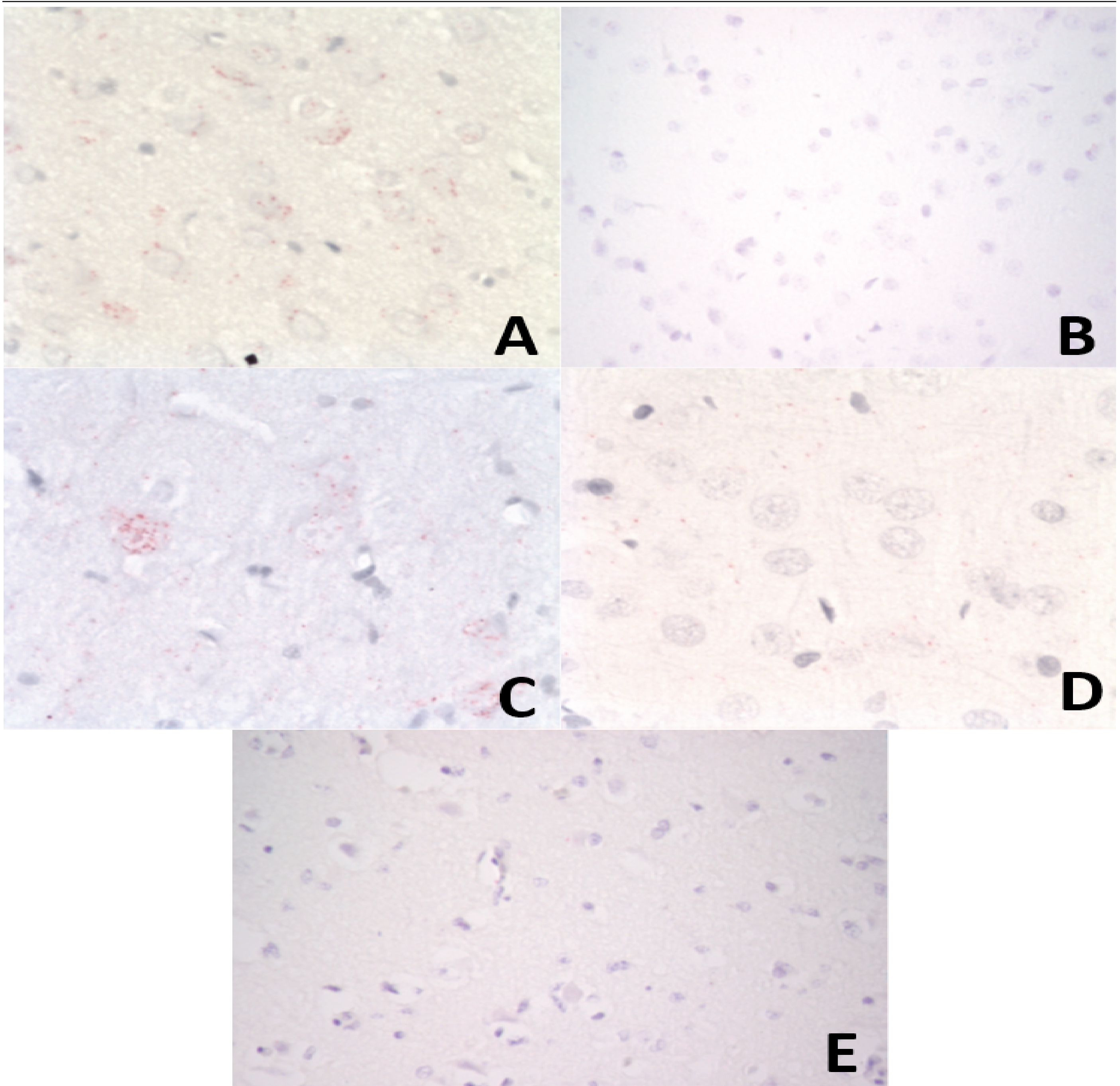

Figure 6: *Visualization of host-virus protein-protein interactions in brain tissues of an experimentally infected rat*
A) *BDV P – HMGB1 interaction (red dots in neuron).* **B)** *BDV P – HMGB1 interaction in an uninfected control rat (no signals).* **C)** *BDV N – Cdc2 interaction (red dots in neurons).* **D)** *BDV N – Cdc2 interaction in an uninfected control rat (no signals).* **E)** *Technical control using primary antibody for HMGB1 only in experimentally infected rat. As expected, no signals are seen due to the need for two primary antibodies to obtain a positive in situ PLA reaction. Magnifications: lens x20 (A, C, D), lens x10 (B, E).*

induction of a pro-inflammatory response. Interactions between BDV P and HMGB1 probably leads to reduced secretion of HMGB1, hence interfering with its important effects through RAGE and TLR4 (Kamitani et al., 2001). In the same study, it was shown that expression of RAGE mRNA was decreased in C6BV cells compared to C6 cells, further indicating that BDV P interferes with HMGB1 and RAGE interactions. Thus, interactions between BDV P and HMGB1 could be a means for BDV to reduce the inflammatory response, thereby evading the host immune response.

Even though it is not possible to draw any conclusions regarding species differences in BDV P-HMGB1 interactions based on this limited study, our findings lead to interesting questions. For example, could it be more important for BDV to interfere with HMGB1 and its pro-inflammatory effects early on in infection, rather than later, when persistence already is established?

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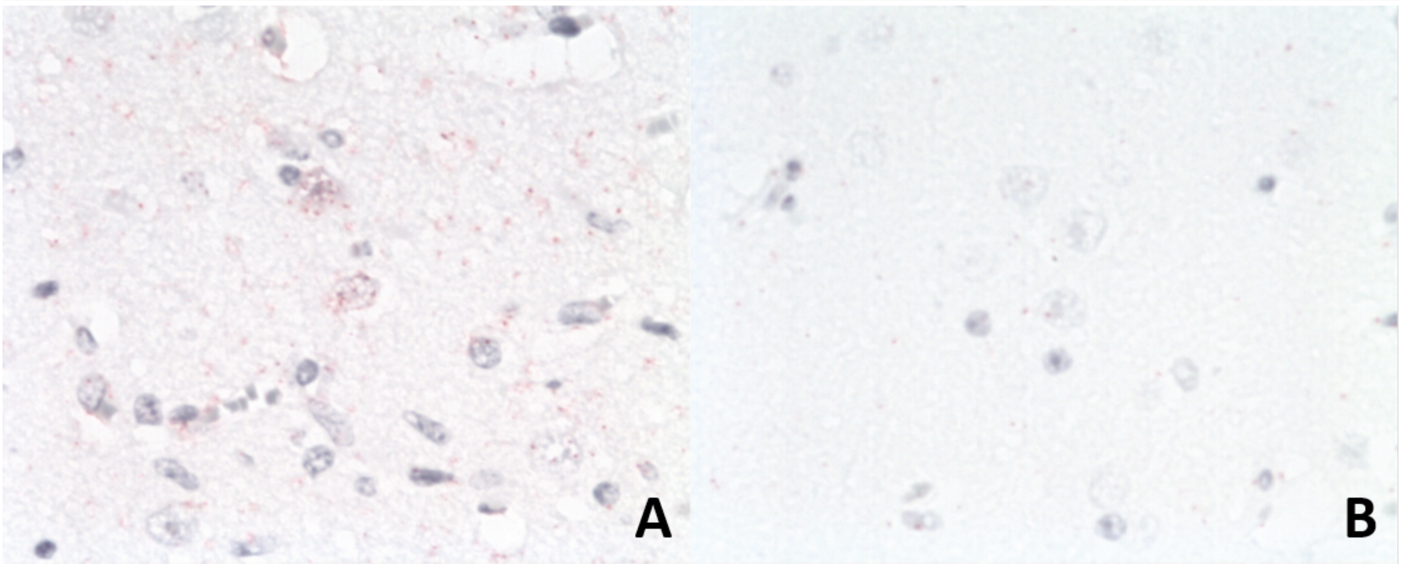

Figure 7: *Visualization of host-virus protein-protein interactions in brain tissues of a naturally infected horse* **A)** *BDV N – Cdc2 interaction (red dots in neurons).* **B)** *BDV N – Cdc2 interaction in an uninfected control horse (no signals). Magnifications: lens x20.*

The infected rat was sacrificed early in infection (day 30 post infection), when BDV is probably still in the process of establishing persistence. The duration of infection in the investigated horse is not known, but it is likely that the horse was euthanized at a later stage of infection than the rat. Different routes of infection could also be of importance. Last but not least, a strain-dependency could also account for this difference, as the rat was infected by a laboratory strain, whereas the horse acquired a natural strain. A recent comparative *in vitro* metabonomic approach using rat cortical neurons infected with either a natural human strain or a laboratory strain revealed divergently altered key energy and amino acid metabolites, pointing to significant biological differences despite low genetic divergence (Liu S et al., 2015).

Interaction between BDV N and Cdc2 was detected in scattered neurons both in the experimentally infected rat and in the naturally infected horse (Figure 6C and 7A), whereas no or only very few signals were found in the uninfected controls (Figure 6D and 7B). Since we cannot distinguish interactions between BDV N or P and Cdc2, we chose to use only anti-BDV N antibodies in tissues. Most likely, Cdc2 also interacts with BDV P in infected animals, although BDV N seems to be crucial for the interference of the activity of Cdc2-Cyclin B1, based on previous *in vitro* studies (Planz et al., 2003).

Cdc2 and Cyclin B1 have been detected in degenerated neurons in tissue from patients with Alzheimer's disease (Vincent et al., 1997), indicating a possible role for this complex in neurological diseases. Moreover, neurogenesis in the dentate gyrus of adult hippocampus, an area targeted for BDV-infection (Gosztonyi and Ludwig, 1995), is an important process (Kempermann et al., 2015), in which Cdc2 plays a role (Jiang and Hsieh, 2014; Uberti et al., 2001). Tissue damage in the CNS likely induces cell cycle activity in neurons, oligodendrocytes and glial cells. Although these interactions here were only seen in neurons, they could nonetheless be of importance also in glial cells. This seems likely, given the demonstrated contrary impact of different BDV strains, either of natural or non-natural origin, on proliferation and apoptosis of infected oligodendroglial (OL) cells (Li et al., 2013). Moreover, a human BDV strain was shown to manipulate histone lysine acetylation and the entire acetylome upon persistence (Liu X et al., 2014; Liu X et al., 2015). Comparing different strains could be a reasonable approach in future studies. In this context, the recently proposed subspecies level classification of BDV type 1 strains may provide a helpful template (Liu S et al., 2015).

Conclusions

In summary, we have used *in situ* PLA to visualize BDV protein and BDV-host protein-protein interactions directly in infected cell cultures and tissues. The overall impact of the studied interactions will require further investigations, especially regarding different virus strains, establishment of persistence and host differences. The presented approach will be of

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further interest to unravel the pathogenesis of various infectious agents both in cell cultures and in experimentally, as well as in naturally infected host species, including man.

Acknowledgements

The authors would like to acknowledge Dr F Södersten, K Vargmar and J Näslund for sampling of non-infected horse brain tissues, and A Boström, Å Gessbo, Dr A-L Blomström and Dr M Munir for excellent technical assistance. This study was funded by the Swedish Foundation of Equine Research (Stiftelsen Svensk Hästforskning; project number H0747211), and supported by the Award of Excellence provided to SB by the Swedish University of Agricultural Sciences and the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013) / ERC Grant Agreement n. 294409 (ProteinSeq) provided to UL.

Conflict of Interests

UL is a co-founder and shareholder of Olink Bioscience, having rights to the *in situ* PLA technology.

Authors' Contributions

JJW conceived, designed and coordinated the study, performed IHC, *in situ* PLA, cell culturing, analyzed and interpreted the data and drafted the manuscript. KJL conceived and designed the study, performed IHC, *in situ* PLA and image analysis, analyzed and interpreted the data, and helped to draft the manuscript. JY performed IHC, *in situ* PLA, cell culturing, and analyzed the data. ALB participated in the design of the study, performed IHC, analyzed and interpreted the data. LB and HL prepared the monoclonal anti-BDV antibodies, interpreted the data and critically revised the manuscript. SB, UL and OS participated in conceiving, designing and supervising the study. MB conceived, designed and supervised the study, analyzed and interpreted the data, and helped to draft the manuscript. All authors read and approved the final manuscript.

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