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# Infection of the enteric nervous system by Borna disease virus (BDV) upregulates expression of Calbindin D-28k

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**Abstract**

Borna disease virus (BDV) is a neurotropic agent infecting distinct neuronal subpopulations in the central nervous system of various mammalian species possibly including humans. Horses, a major natural host for BDV, show gastrointestinal dysfunctions besides characteristic neurological symptoms. Therefore, we hypothesized that enteric neurons may be targets of BDV replication. The presence of BDV-specific antigen in subpopulations of the ENS was investigated. Four-week-old Lewis rats were infected intracerebrally and sacrificed 4–14 weeks post infection (p.i.). BDV-immunoreactive neurons were found in submucous and myenteric neurons of the proximal colon. Fourteen weeks p.i., the proportion of BDV-positive neurons was $44 \pm 17$ and $24 \pm 7\%$ in the submucous and myenteric plexus, respectively. The majority of BDV-positive myenteric neurons showed immunoreactivity for choline acetyltransferase. Expression of Calbindin D-28k (CALB) was found in 96% of submucous and 67% of myenteric BDV-immunoreactive neurons. Additionally, the number of CALB-immunoreactive neurons was significantly higher in the myenteric plexus of infected rats compared to controls. These data indicate that BDV infects specific subpopulations of enteric neurons. Therefore, the ENS might serve as a site for BDV replication and as an immunoprivileged reservoir for BDV. In addition, upregulation of CALB in neurons of the myenteric plexus is probably induced during BDV-infection.


*Keywords:* Borna disease virus; Calbindin; Choline acetyltransferase; Colon; ENS; Lewis rat

## 1. Introduction

Borna disease virus (BDV) is a neurotropic agent infective to a broad range of warmblooded species

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possibly including humans (Staeheli et al., 2000; Lieb and Staeheli, 2001). BDV-infection primarily targets the central nervous system leading to severe neurological symptoms (De la Torre, 2002). Besides infection of the CNS, BDV replicates in peripheral nervous tissues and can migrate centripetally to the CNS (Carbone et al., 1987) or may also move centrifugally from the CNS to peripheral nervous tissues including the enteric nervous system (Carbone et al., 1987; Stitz et al., 1998). Consequently, the peripheral nervous system plays a critical role in the pathogenesis of Borna disease. In horses, the main hosts for BDV, gastrointestinal dysfunctions are described (Bode et al., 1994; Ludwig and Bode, 2000; Richt et al., 2000). Although the extent of BDV-infection in the enteric neurous system has not been determined yet, these gastrointestinal dysfunctions point to direct and/or indirect alterations of the enteric neurones induced by BDV.

Most functions of the gastrointestinal tract are directly influenced by neurons of the enteric nervous system. Their specific control of different target tissues within the GI-tract is ensured by the release of defined combinations of neurotransmitters. The combination of neurotransmitters allows distinguishing functional different classes of enteric neurons according to their neurochemical code (Brookes, 2001). A prominent neurotransmitter mediating excitation of various target tissues is acetylcholine (McConalogue and Furness, 1994). Acetylcholine can be released from excitatory motorneurons, from secretomotorneurons, from some classes of interneurons and from intrinsic primary afferent neurons (McConalogue and Furness, 1994). Cholinergic primary afferent neurons express immunoreactivity for the calcium binding protein Calbindin D-28k (CALB) in some species like guinea pigs and possibly rats (Furness et al., 1998; Mann et al., 1999). This allows differentiation of these neurons from the other cholinergic subpopulations (Furness et al., 1998).

During BDV-infection within the CNS, cholinergic pathways as well as CALB-immunoreactive neuronal subpopulations are altered (Eisenman et al., 1999; Gies et al., 2001; Mayer et al., 2005). However, for the enteric nervous system no data are available concerning the infection of specific neuronal subpopulations by BDV.

In the present study, we therefore examined the appearance of BDV-antigen in the myenteric and submucous plexus of the proximal colon of Lewis rats after intracerebral infection and the number of neurons infected by BDV. Alterations of the enteric neurons during BDV-infection were determined by studying the expression of choline acetyltransferase (ChAT) and CALB.

## 2. Material and methods

### 2.1. Animals

The experiments described in this report comply with the current legislation covering the protection of animals and have been approved by the independent Ethical Committee for Animal Experiments of the Regierungspräsidium Gießen, Az. Gi 18/7-6/95.

The rat-adapted BDV isolate 5/25/92, a derivative of the He/80 strain, was used throughout these studies. This strain was originally isolated from the brain of a BDV-infected horse (Richt et al., 1994). Four-week-old Lewis rats was anaesthesized and BDV was inoculated into the left cerebral hemisphere using 0.1 ml of 10% BDV-brain suspension. Control animals of the same age were mock infected with a 1:10 dilution of uninfected rat brain homogenate in dilution medium. Four, 6, 10, or 14 weeks post infection (p.i.) the rats were anaesthesized by intramuscular injection of ketamin and xylazin and killed by exsanguination. For each time point three infected and three mock-infected animals were used.

### 2.2. Tissue preparation

After exsanguination, the abdomen was opened. The proximal colon was quickly removed and placed in Krebs solution gassed with 95% $O_2$ and 5% $CO_2$. The Krebs solution contained, in mM: NaCl, 117; KCl, 4.7; $MgCl_2$, 1.2; $NaH_2PO_4$, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; glucose, 11.5 and 1 μM nifedipine at pH 7.4.

For whole mount preparations, the tissues were pinned out, mucosa side up, onto silicone (Sylgard, WPI, Germany) covered slides and fixed for 24 h at 4 °C temperature in 0.1 M phosphate buffer containing 4% paraformaldehyde and 0.2% picric acid. After washes with 0.1 M phosphate buffer (3 × 10 min) the tissues were stored in PBS containing 0.1% $NaN_3$.

After removing the epithelium, the submucous plexus was dissected from the circular muscle. Whole mounts of the myenteric plexus were prepared by removing the circular muscle.

Specimens were processed for fluorescence immunohistochemistry as described previously (Schemann et al., 1995). Briefly, after washing and preincubation for 60 min in buffer solution (PBS containing 4% horse serum and 0.5% Triton X-100), the tissue was incubated with combinations of the primary antibodies for 12–16 h at room temperature. The following antisera were used in the respective concentration: rabbit anti-neurone-specific enolase (NSE, 1:3000, 16625, Polysciences, USA), mouse anti-Hu-protein (Hu, 1:200, A21271, MoBiTec, Germany) mouse anti-BDV (1:500 (Haas et al., 1986)), rabbit anti-CALB (1:2000; CB-38, Swant, Switzerland), rabbit anti-ChAT (1:1000, P3YEB (Schemann et al., 1993)). Then the tissue was washed (three times for 10 min) before it was incubated in buffer solution containing the secondary antibodies. Secondary anti-mouse or anti-rabbit IgG raised in donkeys and conjugated to Cy3 or Cy2 (Dianova, Hamburg, Germany) were used. The final dilutions of secondary antibodies were 1:500 (Cy3 conjugates) or 1:200 (Cy2 conjugates), respectively. After the final washing the tissue was mounted on slides in a PBS/glycerol solution and analyzed using an epifluorescence microscope (IX50, Olympus, Japan) attached to an image analysis system (Video camera: Mod. 4910, Cohu Inc., San Diego, California, USA; Macintosh computer and IPLab Spectrum 3.0 software; Signal Analytics, Vienna, Virginia, USA).

### 2.3. Data analysis and statistics

In each tissue 20 submucous or myenteric ganglia, selected randomly, were analyzed. For BDV-, ChAT and CALB-immunoreactivity, the number of cells per ganglion was counted and analyzed in respect to the number of cells per ganglion using NSE- or Hu-immunoreactivity. For each animal, the relative number of BDV-immunoreactive neurons and proportion of CALB- or ChAT-positive subpopulation was calculated. Results are given as mean values ± standard deviation, calculated from three animals of each infected or mock-infected group. Differences were considered as statistically significant at values of $p < 0.05$. Proportion of neuronal subpopulations after different infection times was compared using a one-way ANOVA with subsequent multiple comparisons (Student–Newman–Keuls test). Student's *t*-test was used to compare the proportion of one subpopulation between infected rats and control rats or between BDV-positive and -negative neurons. Two-way ANOVA with subsequent multiple comparisons (Student–Newman–Keuls test) was used for comparison of NSE (or Hu), ChAT, CALB or BDV immunoreactivity after different infection times (Factor A) and between infected rats and control rats, or submucous and myenteric plexus (Factor B).

### 2.4. PCR

For detection of BDV-specific mRNA, plexus samples were stored at −70 °C. After 3 × 15 s sonification RNA-isolation was carried out using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions. BDV p40 specific one-step RT-PCR was performed using the QIAGEN OneStep RT-PCR kit (Qiagen, Hilden, Germany). The RNA was first incubated at 72 °C for 3 min to denature the RNA, followed by 3 min cooling on ice. A total of 20 μl of the RNA solution was used in a 50 μl reaction. The reaction contained 100 pmol each of primers p40s (5′-ACGCC-CAGCCTTGTGTTTCT-3′) and p40 as (5′-AATTCTTTACCTGGGGACTCAA-3′), to amplify a 449 bp fragment of the genome (approximately nucleotides 270–720). Reactions were set up according to the supplier's recommendation. After reaction setup, reverse transcription was carried out for 30 min at 50 °C. Then a hot start of 15 min at 95 °C was included to activate HotStarTaq™ DNA Polymerase in the reaction mix. PCR followed immediately, with 45 cycles of 94 °C for 30 s, 56.5 °C for 45 s, and 72 °C for 1 min. The final extension step consisted of 10 min at 72 °C. Subsequently, a BDV p40 specific nested PCR amplifying a 281 bp fragment was performed as described previously (Vahlenkamp et al., 2000).

### 2.5. Restriction enzyme analysis (REA)

For specification by REA, another 5 μl aliquot of the nested PCR products was digested with 5 U NcoI in a final volume of 20 μl according to the instructions of the supplier (New England Biolabs, Frankfurt a. M.,

Germany). In REA, electrophoresis was carried out on 2% ethidium bromide-stained agarose gels; fragments were visualized by UV transillumination.

## 3. Results

In infected rats clinical symptoms of gastrointestinal dysfunctions were not obvious. By using the panneuronal markers NSE and Hu, the number of neurons per ganglion was counted in the submucous and myenteric plexus of infected and control rats. The number of neurons was compared using two-way ANOVA (Factor 1: time point of infection, Factor 2: infected animal or control animal) with subsequent Student–Newman–Keuls test. For each time point, tissues from three infected rats and three control rats were analyzed. In the submucous plexus of infected rats, ganglia contained $9 \pm 1$ neurons/ganglion 4 weeks post infection (p.i.). In the course of infection, the amount of neurons/ganglion was not significantly altered ($10 \pm 1$ neurons/ganglion at 14 weeks p.i.).

In control rats, the number of neurons per ganglion in the submucous plexus was similar to that in infected

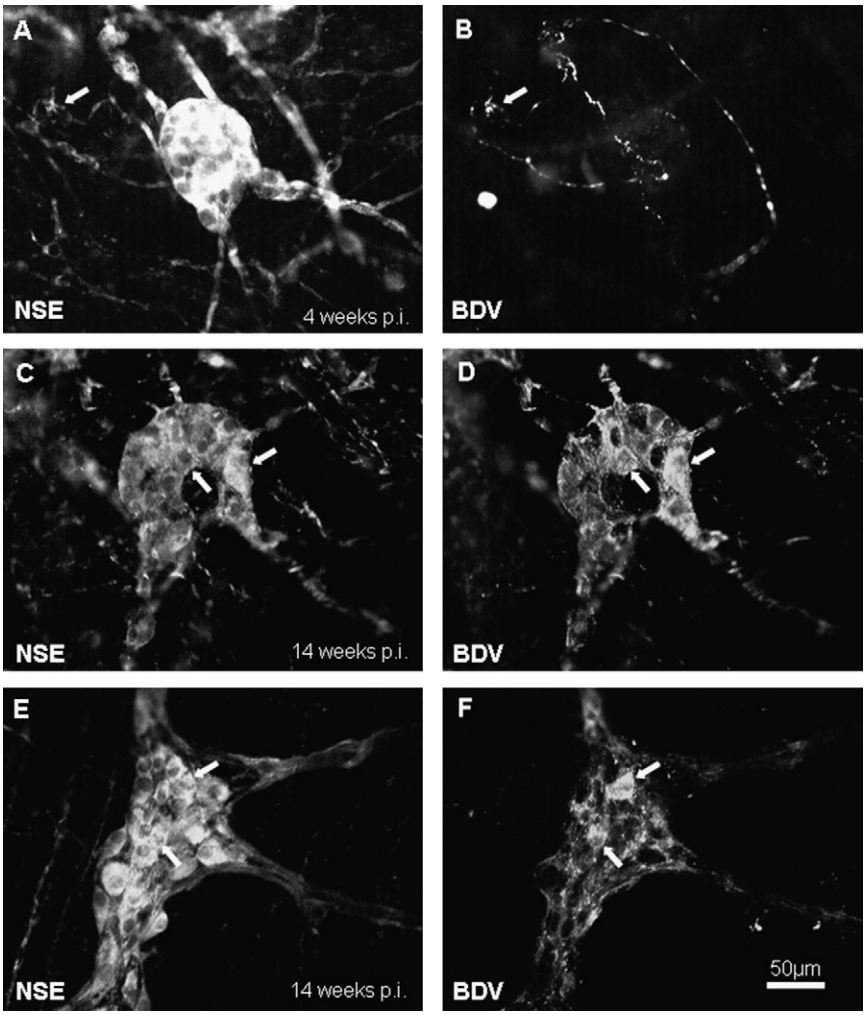


Fig. 1. Photomicrographs showing the presence of BDV-specific antigen in the submucous (A–D) and myenteric (E and F) plexus. (A and B) In the majority of submucous ganglia, only nerve fibres (indicated by an arrow) are BDV-immunoreactive 4 weeks p.i. After 14 weeks p.i., BDV-immunoreactive somata (examples are indicated by arrows) can be found in the submucous (C and D) and myenteric (E and F) plexus.

rats ($8 \pm 1$ neurons/ganglion at 4 weeks post mock infection (p.m.i.), $10 \pm 2$ at 14 week p.m.i.).

Myenteric ganglia of BDV-infected rats contained $23 \pm 0.3$ neurons/ganglion at 4 weeks p.i. As for the submucous plexus, the value was not significantly changed during the course of infection. In contrast to submucosal ganglia, number of neurons/ganglion in myenteric ganglia was higher in control rats at 4 and 6 weeks p.m.i. ($30 \pm 3$ at 4 weeks p.m.i. and $27 \pm 2$ at 6 weeks p.m.i.) than in BDV-infected rats. At 10 and 14 weeks p.i., size of myenteric ganglia was similar in BDV-infected and mock-infected rats.

### 3.1. Presence of BDV in enteric neurons

Immunoreactivity for BDV was found exclusively in gastrointestinal tissues of infected rats, but not in gastrointestinal tissues of control rats. Co-staining of BDV and NSE revealed that only neurons and nerve fibres in the submucous and myenteric plexus were immunoreactive for BDV (Fig. 1).

The presence of BDV was confirmed by nested PCR with subsequent REA using NcoI. NcoI has one cleavage site in the p40 specific nested PCR product. Digestion of the PCR products of the plexus samples revealed fragments with the expected length of 178 and 103 bp.

In the submucous plexus, only in the tissues of one of three infected animals scarce BDV-immunoreactivity could be found at 4 weeks p.i. (Fig. 1A and B). At later stages of infection, BDV-immunoreactivity could be found in all preparations of the submucous plexus from infected animals (three animals for each time point). The relative portion of BDV-immunoreactive neurons increased significantly to $39.9 \pm 8.9\%$ of all submucous neurons from 4 to 6 weeks p.i. and tended ($p = 0.075$) to increase further up to 10 weeks (Fig. 2).

In the myenteric plexus of all infected animals, BDV-immunoreactivity could be demonstrated. Four weeks p.i. $10.8 \pm 6.8\%$ of neurons expressed BDV-immunoreactivity. At 14 weeks p.i., the proportion was $23.5 \pm 7.1\%$ of total myenteric neurons (Fig. 2).

Comparing the relative number of BDV-infected neurons between the submucous and myenteric plexus, the proportion of BDV-positive neurons was significantly higher in the submucous plexus at 6 and 10 weeks p.i. (Fig. 2).

### 3.2. Neurochemistry of BDV-positive neurons

BDV-positive and -negative neurons were examined for their immunoreactivity for ChAT and CALB (see Figs. 3 and 4). Four weeks p.i., BDV-immunoreactive neurons were found in the submucous plexus of only one animal. Therefore, submucous neurons were examined obtained from animals 6, 10 and 14 weeks p.i. (three animals for each time point). Myenteric neurons were examined obtained from animals 4, 6, 10 and 14 weeks p.i. (three animals for each time point).

During the course of infection, the relative number of ChAT- and CALB-immunoreactive neurons did neither change in the submucous, nor in the myenteric plexus (compared with one-way ANOVA). Therefore, we pooled the data from the different infection periods

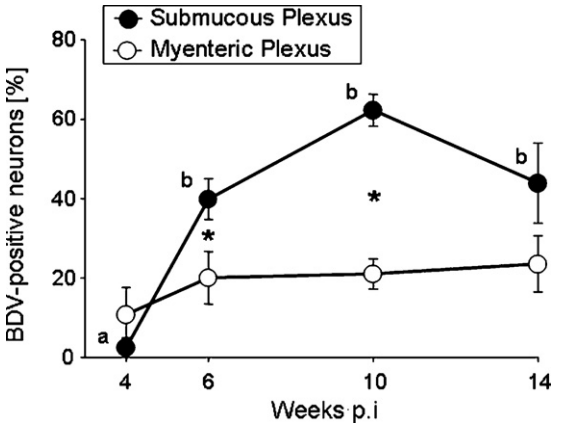


Fig. 2. Proportion of BDV-positive neurons in the submucous (filled circles) and myenteric (open circles) plexus of infected animals. Results were calculated from analysis of 10062 myenteric and 4209 submucous neurones from 24 myenteric and 24 submucous plexus preparations of 12 animals. The preparations were stained using antibodies against the general neuronal marker NSE and against BDV. Relative number of submucous neurons infected with BDV increased up to 6 weeks p.i. and tended ($p = 0.075$) to further increase up to 10 weeks p.i. Proportion of BDV-positive myenteric neurons did not differ significantly between the different infection times. Asterisks indicate significant differences ($p < 0.05$, Student's *t*-test) between the submucous and myenteric plexus. (a and b) Different letters indicate significant differences ($p < 0.05$, one-way ANOVA with subsequent Student–Newman–Keuls test) in the submucous plexus between different infection times.

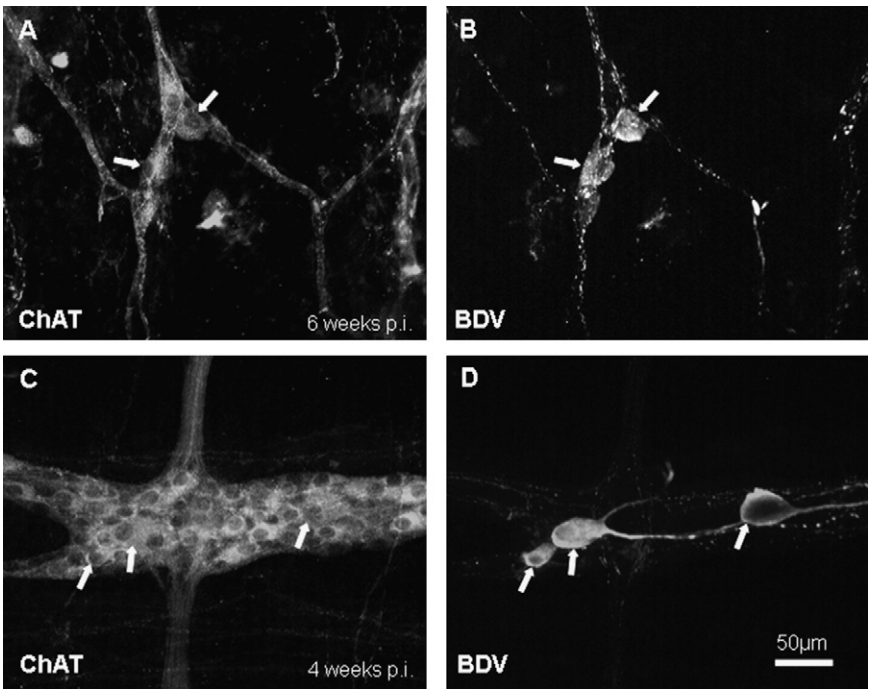


Fig. 3. Photomicrographs showing a submucous (A and B) and a myenteric (C and D) ganglion 6 and 4 weeks p.i., respectively. Tissues were double labelled against ChAT (A and C) and BDV (B and D). The vast majority of BDV-positive neurons express immunoreactivity for ChAT (examples are indicated by arrows).

for the submucous and the myenteric plexus for further comparisons.

### 3.3. ChAT-immunoreactive populations

By analyzing the immunoreacitvity for ChAT and BDV, the following subpopulations could be distinguished: BDV-positive and ChAT-positive neurons (BDV+/ChAT+), BDV-negative, ChAT-positive neurons (BDV−/ChAT+), BDV-positive and ChAT-negative neurons (BDV+/ChAT−) and neurons not immunoreactive for BDV or ChAT (BDV−/ChAT−). In the submucous plexus, these four subpopulations were present in comparable numbers (Fig. 5). In the myenteric plexus the relative portions of these subpopulations were as follows: BDV−/ChAT+ > BDV−/ChAT− > BDV+/ChAT+ > BDV+/ChAT− (Fig. 5).

To examine if BDV primarily targets ChAT-positive neurons, the proportion of ChAT-positive neurons was compared between the BDV-positive and the BDV-negative population in infected rats. Analyzing the data revealed that $57.8 \pm 21.2\%$ of BDV-immunoreactive neurons were immunoreactive also for ChAT in the submucous plexus of BDV-infected animals (Table 1). A similar proportion of ChAT-positive neurons were found in the population of BDV-negative neurons of BDV-infected animals (Table 1).

In contrast to findings from the submucous plexus, BDV-positive myenteric neurons expressed ChAT-immunoreactivity to a higher extent than BDV-negative myenteric neurons (Table 1).

Additionally, we analyzed the proportion of BDV-positive neurones in the populations of ChAT-positive and ChAT-negative neurons in infected rats. By using this approach, we found that the proportion of BDV-positive myenteric neurons was significantly higher in the population of ChAT-positive than in the population of ChAT-negative neurons (Table 2). In the submucous plexus of infected rats such differences could not be detected.

To estimate if BDV-infection alters the proportion of ChAT-positive neurons the relative number of

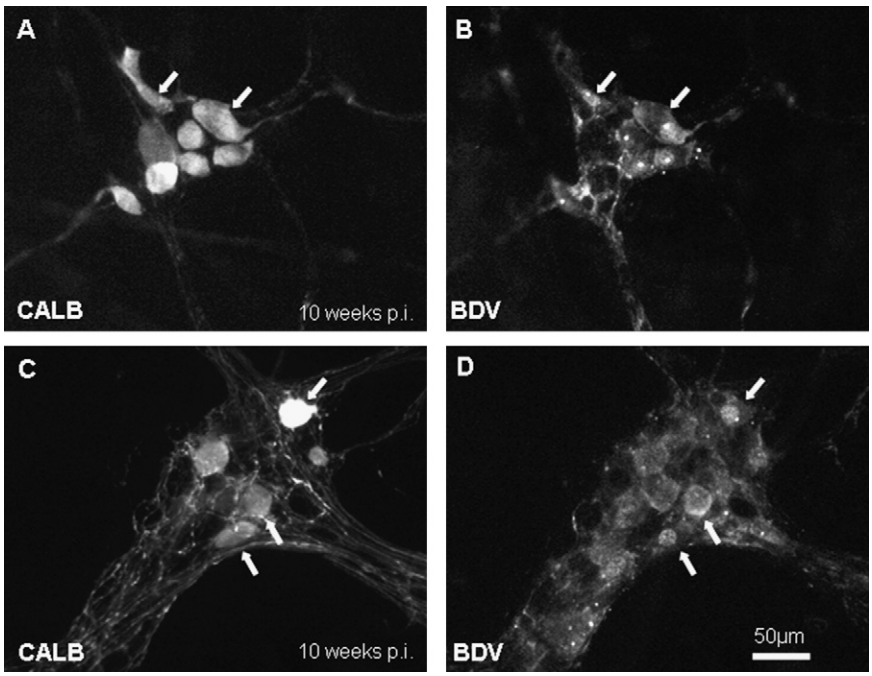


Fig. 4. Photomicrographs showing CALB-immunoreactivity in BDV-positive submucous (A and B) and myenteric (C and D) neurons 10 weeks post infection. Tissues were double labelled against CALB (A and C) and BDV (B and D). Arrows indicate some neurons which are immunoreactive for both, CALB and BDV.

ChAT-positive neurons between infected animals and control rats was also compared.

It was found that the proportion of ChAT-positive neurons was similar in the submucous as well as in the myenteric plexus of infected rats and control rats (Table 1).

### 3.4. CALB-immunoreactive populations

Examining the immunoreactivity for BDV and CALB and subsequent classification revealed the following subpopulations: BDV-positive and CALB-positive neurons (BDV+/CALB+), BDV-negative and CALB-positive neurons (BDV−/CALB+), BDV-positive and CALB-negative neurons (BDV+/CALB−) and neurons not immunoreactive for BDV or CALB (BDV−/CALB−). CALB-immunoreactive populations in the submucous plexus were larger than CALB-negative populations (Fig. 5). Population sizes of subpopulations in the myenteric plexus were as follows: BDV−/CALB− > BDV−/CALB+ = BDV+/CALB+ > BDV+/CALB− (Fig. 5).

Comparing the proportion of CALB-positive neurons between the BDV-positive and the BDV-negative population of neurons in infected rats, we found that 96.2 ± 7.7% of BDV-positive submucous neurons were immunoreactive for CALB. This proportion was not significantly different from the proportion in the population of the BDV-negative neurons (Table 1). In the myenteric plexus of BDV-infected rats, however, CALB-immunoreactivity was expressed to a higher degree in the population of BDV-positive neurons than in the BDV-negative population (Table 1).

Comparing the proportion of BDV-positive neurons between the populations of CALB-positive and CALB-negative neurons in infected rats, we found that, the fraction of BDV-positive neurons in the population of CALB-positive myenteric neurons was larger than in the population of CALB-negative myenteric neurons (Table 2). In the submucous plexus such difference could not be detected.

During course of infection, the proportion of CALB-immunoreactive neurons did not change.

However, we found a significant higher proportion of CALB-positive neurons in the myenteric plexus of BDV-infected rats as compared to control rats (Table 1). In the submucous plexus, the relative number of CALB-immunoreactive neurons was similar between infected rats and control rats (Table 1).

## 4. Discussion

This study showed that after intracerebral infection of Lewis rats, BDV-immunoreactivity can be found in neurons of the enteric nervous system (ENS). Within the ENS, submucous neurons became BDV-immunoreactive to a higher extend than myenteric neurons. In addition, BDV seemed to replicate in specific subpopulations of neurons which express immunoreactivity for ChAT and CALB, and the relative number of CALB-immunoreactive neurons was higher in the myenteric plexus of infected animals as compared to non-infected.

BDV is a neurotropic virus known to replicate in neurons of the central nervous system (De la Torre, 2002). The route of infection is suggested to be via the olfactory nerve (Sauder and Staeheli, 2003), although other routes like hematogenous transmission are also discussed (Hornig et al., 2003). After colonizing the brain, the virus infection spreads centrifugally into the periphery (Carbone et al., 1987). The latter authors (Carbone et al., 1987) have also shown that BDV is present in peripheral nerves including neurons of the enteric nervous system after intracerebral BDV-infection. Our study confirms and significantly extends these results.

We report that submucous neurons are BDV-positive to a higher extent than myenteric neurons. This might be due to two reasons. Firstly, extrinsic nerve fibres might innervate the submucous plexus to a higher degree than the myenteric plexus and therefore offer a higher possibility to be infected by centrifugally spreading BDV. Secondly, submucous neurons may be more susceptible to BDV-infection than myenteric ones.

The gastrointestinal tract is connected to higher centres via the parasympathetic and sympathetic nervous system and via spinal afferent neurons (Brookes and Costa, 2006). In the proximal colon, the prominent extrinsic input originates from sympathetic fibres (Brookes and Costa, 2006). Sympathetic ganglia as well as dorsal root ganglia have been found to be a route for BDV spread after intracerebral infection (Carbone et al., 1987). Although extrinsic colonic innervation was not investigated in the present study, it seems likely that the virus entered the enteric nervous system via sympathetic neurons or via spinal afferent fibres.

Sympathetic fibres as well as spinal afferents terminate in the myenteric plexus as well as in submucous ganglia (Patterson et al., 2003; Brookes

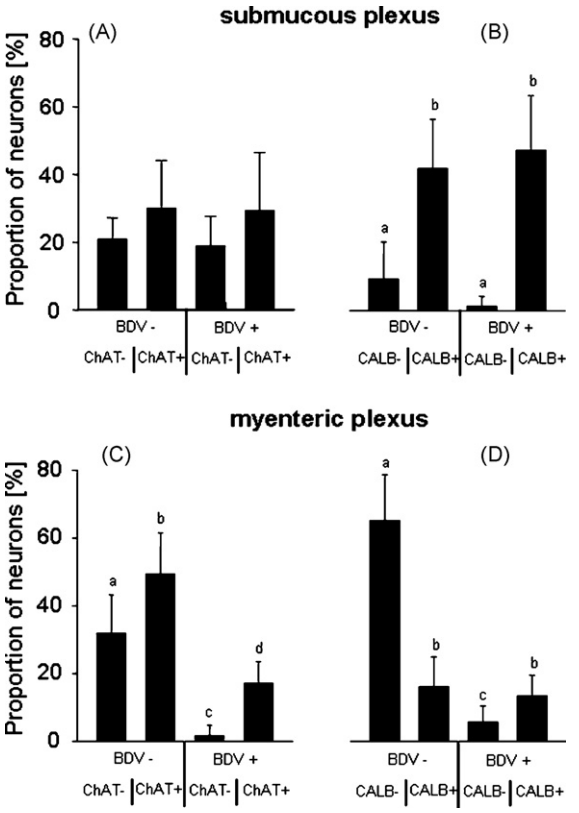


Fig. 5. Relative amount of subpopulations in the submucous (A and B) and myenteric plexus (C and D) of BDV-infected rats. Data from 9 (6–14 weeks p.i.) and 12 (4–14 weeks p.i.) rats are pooled for the submucous and myenteric plexus, respectively. The bar charts A and C show subpopulations revealed by staining against NSE, BDV and ChAT. Analysis of immunoreactivity for NSE, BDV and CALB revealed subpopulations shown in the bar charts B and D. (A–D) Columns within each graph are significantly different ($p < 0.05$, one-way ANOVA with subsequent Student–Newman–Keuls test) if they do not share a common letter; no significant differences were found between the subpopulations shown in A.

Table 1
Expression of ChAT and CALB in infected animals and control animals

| | Population set as 100% | ChAT+ (%) | CALB+ (%) |
|---|---|---|---|
| SMP | All neurons of infected animals | 59.3 ± 11.7 | 90.5 ± 10.1 |
| | All neurons of control animals | 53.4 ± 9.9 | 84.1 ± 8.5 |
| MP | All neurons of infected animals | 66.3 ± 10.3 | 29.3 ± 14.2* |
| | All neurons of control animals | 56.9 ± 6.0 | 14.7 ± 3.7 |
| SMP | BDV-positive neurons of infected animals | 57.8 ± 21.2 | 96.2 ± 7.7 |
| | BDV-negative neurons of infected animals | 58.8 ± 15.9 | 82.0 ± 21.3 |
| MP | BDV-positive neurons of infected animals | 91.8 ± 11.5* | 66.6 ± 28.4* |
| | BDV-negative neurons of infected animals | 60.6 ± 14.1 | 20.3 ± 11.7 |

ChAT- and CALB-immunoreactivity in the overall population of neurons in infected animals and control animals and in BDV-positive and -negative neurons of infected animals was compared. Data have been obtained from 9 and 12 infected animals for the submucous and myenteric plexus, respectively, and from 12 control animals. Tissues were stained either against ChAT or against CALB in combination with staining against BDV or Hu. (*) Asterisks indicate significant differences ($p < 0.05$, $t$-test) between infected animals and control animals or between the populations of BDV-positive or -negative neurons.

and Costa, 2006). This is in accordance with the observed appearance of BDV-antigen in both submucous and myenteric neurons at similar points in time p.i. However, no data are available at present regarding the extent of projection of sympathetic fibres and spinal afferents to submucous and myenteric ganglia. Consequently, it is possible that the higher amount of BDV-antigens in the submucous than in the myenteric plexus is due to a denser extrinsic innervation of submucous neurons. However, supporting data for this hypothesis are still lacking.

Besides the centripetal BDV transmission via the olfactory nerve to the CNS (Hornig et al., 2003) and subsequent centrifugal spread, an oronasal infection may also directly target enteric neurons projecting to the gastrointestinal epithelium. Presence of BDV in the gastrointestinal tract can be presumed, since in persistently infected animals BDV can be found in the feces (Sierra-Honigmann et al., 1993). If the assumption is true that BDV-infection enters the body via the enteric nervous system, virus replication in neurons innervating the epithelium, i.e. submucous neurons is likely. However, so far no data are available concerning the susceptibility of submucous and myenteric neurons to BDV replication. However, Carbone et al. (1987) could not demonstrate an oral BDV-infection with subsequent virus transmission via the enteric nervous system.

Table 2
Immunoreactivity for BDV in ChAT-positive and CALB-positive neurons

| | Population set as 100% | BDV+ (%) |
|---|---|---|
| SMP | ChAT-positive neurons of infected animals | 48.8 ± 24.4 |
| | ChAT-negative neurons of infected animals | 46.0 ± 15.4 |
| MP | ChAT-positive neurons of infected animals | 26.5 ± 11.6* |
| | ChAT-negative neurons of infected animals | 5.3 ± 9.2 |
| SMP | CALB-positive neurons of infected animals | 39.6 ± 27.8 |
| | CALB-negative neurons of infected animals | 13.2 ± 27.3 |
| MP | CALB-positive neurons of infected animals | 43.9 ± 16.4* |
| | CALB-negative neurons of infected animals | 7.7 ± 7.0 |

Percentage of BDV-immunoreactive neurons was compared between the populations of ChAT-positive or -negative neurons, or between the populations of CALB-positive or -negative neurons. Data have been obtained from 9 and 12 infected animals for the submucous and myenteric plexus, respectively, and from 12 control animals. Tissues were stained either against ChAT or against CALB in combination with staining against BDV or Hu. (*) Asterisks indicate significant differences ($p < 0.05$, $t$-test) between the populations of ChAT-positive or -negative neurons or between the populations of CALB-positive or -negative neurons.

In our study, cholinergic subpopulations were the primary target for BDV replication. This finding is consistent with studies obtained in the CNS of rats, where the cholinergic system is also a main target of BDV-infection (Gies et al., 2001). As a consequence, the number of cholinergic neurons in the CNS decreases during BDV-infection (Gies et al., 2001). These changes cannot be found in the cholinergic system of the ENS. In our study, the number of submucous and myenteric neurons as well as the proportion of ChAT-positive neurons remained unchanged during BDV-infection. Therefore, it seems unlikely that BDV induces gastrointestinal dysfunctions by disturbing some enteric neuronal subpopulations in Lewis rats. To date, gastrointestinal dysfunctions during BDV-infection have been described only in horses (Bode et al., 1994; Ludwig and Bode, 2000; Richt et al., 2000), suggesting species differences of BDV-induced morphological alteration of subpopulations in the ENS. Further studies are required to test whether the function of enteric neurons is altered during BDV-infection in horses.

In addition to cholinergic neurons, neurons immunoreactive for the CALB are susceptible for BDV-infection in the CNS (Eisenman et al., 1999; Mayer et al., 2005). A selective loss of CALB-immunoreactive hippocampal neurons can be found during BDV-infection (Mayer et al., 2005). Unlike the findings from the CNS, BDV seems to induce an upregulation of CALB in neurons particularly in the myenteric plexus.

In the myenteric plexus of BDV-infected rats, 29% of neurons expressed immunoreactivity for CALB. In control rats the portion of CALB-immunoreactive neurons was only 15%. Such an alteration could not be detected in the submucous plexus. However, regarding the submucous plexus it must be emphasized that about 84% of submucous neurons expressed CALB-immunoreactivity in control rats. An upregulation of CALB-immunoreactivity would therefore possibly be masked by inter-individual variances in the proportion of CALB-positive neurons.

It remains unclear if CALB-expression was generally upregulated in myenteric neurons of BDV-infected rats, or if upregulation was restricted to neurons which were directly infected by BDV. Analyzing the proportion of CALB-positive neurons in myenteric subpopulations of infected rats, we found 15% of myenteric neurons to be BDV-negative and CALB-positive. This relative number (15%) of CALB-positive neurons can be also found in the myenteric plexus of control rats. Therefore, it seems likely that BDV does not specifically infect CALB-positive neurons, but BDV-infection induces the expression of CALB. In this regard, function of CALB is still not clear. However, recent data indicate that CALB plays a major role in different cell types in protecting against apoptotic cell death (Christakos et al., 2003). Apoptosis of central neurons is also caused by BDV-infection in neonatally infected rats (Hornig et al., 1999; Weissenbock et al., 2000). Consequently, the upregulation of CALB-expression in the ENS might be a protective mechanism to prevent neurons from cell death.

In conclusion, we found BDV to replicate in cholinergic neurons of the enteric nervous system. In contrast to observations on CNS neurons, BDV-infection did not cause a loss of enteric neurons but induced an upregulation of CALB-expression in the myenteric plexus. It has still to be elucidated, if CALB-upregulation is a mechanism of neuroprotection. Further studies in other species are required to proof the significance of our results obtained in rats.

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