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1 **Infection of the enteric nervous system by Borna Disease Virus**
2 **(BDV) upregulates expression of Calbindin D-28k**

3

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12

13 **Running title:** BDV in the ENS

14

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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.

Key words: Borna Disease Virus, calbindin, choline acetyltransferase, colon, ENS, Lewis rat

48

49 Introduction

50 Borna disease virus (BDV) is a neurotropic agent infective to a broad range of
51 warmblooded species possibly including humans (Staeheli et al., 2000; Lieb and
52 Staeheli, 2001). BDV infection primarily targets the central nervous system leading to
53 severe neurological symptoms (De la Torre, 2002). Besides infection of the CNS,
54 BDV replicates in peripheral nervous tissues and can migrate centripetally to the
55 CNS (Carbone et al., 1987) or may also move centrifugally from the CNS to
56 peripheral nervous tissues including the enteric nervous system (Carbone et al.,
57 1987; Stitz et al., 1998). Consequently, the peripheral nervous system plays a critical
58 role in the pathogenesis of Borna disease. In horses, the main hosts for BDV,
59 gastrointestinal dysfunctions are described (Bode et al., 1994; Ludwig and Bode,
60 2000; Richt et al., 2000). Although the extent of BDV-infection in the enteric nervous
61 system has not been determined yet, these gastrointestinal dysfunctions point to
62 direct and/or indirect alterations of the enteric neurones induced by BDV.

63 Most functions of the gastrointestinal tract are directly influenced by neurons of the
64 enteric nervous system. Their specific control of different target tissues within the GI-
65 tract is ensured by the release of defined combinations of neurotransmitters. The
66 combination of neurotransmitters allows distinguishing functional different classes of
67 enteric neurons according to their neurochemical code (Brookes, 2001). A prominent
68 neurotransmitter mediating excitation of various target tissues is acetylcholine
69 (McConalogue and Furness, 1994). Acetylcholine can be released from excitatory
70 motoneurons, from secretomotoneurons, from some classes of interneurons and
71 from intrinsic primary afferent neurons (McConalogue and Furness, 1994).
72 Cholinergic primary afferent neurons express immunoreactivity for the calcium
73 binding protein Calbindin D-28k (CALB) in some species like guinea pigs and

74 possibly rats (Furness et al., 1998; Mann et al., 1999). This allows differentiation of
75 these neurons from the other cholinergic subpopulations (Furness et al., 1998).

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

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

85

86 **Material and Methods**

87 **Animals**

88 The experiments described in this report comply with the current legislation covering
89 the protection of animals and have been approved by the independent Ethical
90 Committee for Animal Experiments of the Regierungspräsidium Gießen, Az. Gi 18/7-
91 6/95.

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

99 xylazin and killed by exsanguination. For each time point 3 infected and 3 mock-
100 infected animals were used.

101

102 **Tissue preparation**

103 After exsanguination, the abdomen was opened. The proximal colon was quickly
104 removed and placed in Krebs solution gassed with 95% O₂ and 5% CO₂. The Krebs
105 solution contained, in mM: NaCl: 117 , KCl: 4.7, MgCl₂: 1.2 , NaH₂PO₄: 1.2,
106 NaHCO₃: 25, CaCl₂: 2.5, glucose: 11.5 and 1 μM nifedipine at pH 7.4.

107 For whole mount preparations, the tissues were pinned out, mucosa side up, onto
108 silicone (Sylgard, WPI, Germany) covered slides and fixed for 24 hours at 4°C
109 temperature in 0.1 M phosphate buffer containing 4% paraformaldehyde and 0.2%
110 picric acid. After washes with 0.1 M phosphate buffer (3x10 min) the tissues were
111 stored in PBS containing 0.1% NaN₃.

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

115 Specimens were processed for fluorescence immunohistochemistry as described
116 previously (Schemann et al., 1995). Briefly, after washing and preincubation for 60
117 min in buffer solution (PBS containing 4% horse serum and 0.5% Triton X-100), the
118 tissue was incubated with combinations of the primary antibodies for 12-16 h at room
119 temperature. The following antisera were used in the respective concentration: rabbit
120 anti-neurone-specific enolase (NSE, 1:3000, 16625, Polysciences, USA), mouse anti
121 Hu-protein (Hu, 1:200, A21271, MoBiTec, Germany) mouse anti-BDV (1:500, (Haas
122 et al., 1986)), rabbit anti-CALB (1:2000; CB-38, Swant, Switzerland), rabbit anti-
123 ChAT (1:1000, P3YEB, (Schemann et al., 1993)). Then the tissue was washed (3
124 times for 10 min) before it was incubated in buffer solution containing the secondary

125 antibodies. Secondary anti-mouse or anti-rabbit IgG raised in donkeys and
126 conjugated to Cy3 or Cy2 (Dianova, Hamburg, Germany) were used. The final
127 dilutions of secondary antibodies were 1:500 (Cy3 conjugates) or 1:200 (Cy2
128 conjugates), respectively. After the final washing the tissue was mounted on slides in
129 a PBS/glycerol solution and analysed using an epifluorescence microscope (IX50,
130 Olympus, Japan) attached to an image analysis system (Video camera: Mod. 4910,
131 Cohu Inc., San Diego, California, USA; Macintosh computer and IPLab Spectrum 3.0
132 software; Signal Analytics, Vienna, Virginia, USA).

133

134 **Data analysis and statistics**

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

151

152 **PCR**

153 For detection of BDV specific mRNA, plexus samples were stored at -70 °C. After
154 3x15 sec sonification RNA-isolation was carried out using the RNeasy Mini Kit
155 (Qiagen, Hilden, Germany), according to manufacturer's instructions. BDV p40
156 specific one-step RT-PCR was performed using the QIAGEN OneStep RT-PCR kit
157 (Qiagen, Hilden, Germany). The RNA was first incubated at 72 °C for 3 minutes to
158 denature the RNA, followed by 3 minutes cooling on ice. A total of 20 µl of the RNA
159 solution was used in a 50 µl reaction. The reaction contained 100 pmol each of
160 primers p40s (5' - ACGCCAGCCTTGTGTTTCT-3') and p40 as (5'-
161 AATTCTTTACCTGGGGACTCAA-3'), to amplify a 449 bp fragment of the genome
162 (approximately nucleotides 270-720). Reactions were set up according to the
163 supplier's recommendation. After reaction setup, reverse transcription was carried
164 out for 30 minutes at 50 °C. Then a hot start of 15 minutes at 95 °C was included to
165 activate HotStarTaq™ DNA Polymerase in the reaction mix. PCR followed
166 immediately, with 45 cycles of 94 °C for 30 seconds, 56.5 °C for 45 seconds, and 72
167 °C for 1 minute. The final extension step consisted of 10 minutes at 72 °C.
168 Subsequently a BDV p40 specific nested PCR amplifying a 281 bp fragment was
169 performed as described previously (Vahlenkamp et al., 2000).

170

171 **Restriction enzyme analysis (REA)**

172 For specification by REA, another 5 µl aliquot of the nested PCR products was
173 digested with 5 U *Nco*I in a final volume of 20 µl according to the instructions of the
174 supplier (New England Biolabs, Frankfurt a. M., Germany). In REA, electrophoresis
175 was carried out on 2% ethidium bromide-stained agarose gels; fragments were
176 visualized by UV transillumination.

177 **Results**

178 In infected rats clinical symptoms of gastrointestinal dysfunctions were not obvious.
179 By using the pan-neuronal markers NSE and Hu, the number of neurons per
180 ganglion was counted in the submucous and myenteric plexus of infected and control
181 rats. The number of neurons was compared using two way ANOVA (factor 1: time
182 point of infection, factor 2: infected animal or control animal) with subsequent
183 Student-Newman-Keuls test. For each time point, tissues from 3 infected rats and 3
184 control rats were analyzed. In the submucous plexus of infected rats, ganglia
185 contained 9 ± 1 neurons/ganglion 4 weeks post infection (p.i.). In the course of
186 infection, the amount of neurons/ganglion was not significantly altered (10 ± 1
187 neurons/ganglion at 14 weeks p.i.).

188 In control rats, the number of neurons per ganglion in the submucous plexus was
189 similar to that in infected rats (8 ± 1 neurons per ganglion at 4 weeks post mock-
190 infection (p.m.i.), 10 ± 2 at 14 week p.m.i.).

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

198

199 ***Presence of BDV in enteric neurons***

200 Immunoreactivity for BDV was found exclusively in gastrointestinal tissues of infected
201 rats, but not in gastrointestinal tissues of control rats. Co-staining of BDV and NSE

202 revealed that only neurons and nerve fibres in the submucous and myenteric plexus
203 were immunoreactive for BDV (Fig. 1).

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

208

209 In the submucous plexus, only in the tissues of one of 3 infected animals scarce
210 BDV-immunoreactivity could be found at 4 weeks p.i. (Fig. 1A, B). At later stages of
211 infection, BDV-immunoreactivity could be found in all preparations of the submucous
212 plexus from infected animals (3 animals for each time point). The relative portion of
213 BDV-immunoreactive neurons increased significantly to $39.9 \pm 8.9\%$ of all submucous
214 neurons from 4 to 6 weeks p.i. and tended ($p=0.075$) to increase further up to 10
215 weeks (Fig. 2).

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

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

223

224 ***Neurochemistry of BDV-positive neurons***

225 BDV-positive and -negative neurons were examined for their immunoreactivity for
226 ChAT and CALB (see Figs. 3, 4). Four weeks p.i., BDV-immunoreactive neurons
227 were found in the submucous plexus of only one animal. Therefore, submucous

228 neurons were examined obtained from animals 6, 10 and 14 weeks p.i. (3 animals for
229 each time point). Myenteric neurons were examined obtained from animals 4, 6, 10
230 and 14 weeks p.i. (3 animals for each time point).

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

236

237 *ChAT-immunoreactive populations*

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

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

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

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

262 To estimate if BDV-infection alters the proportion of ChAT-positive neurons the
263 relative number of ChAT-positive neurons between infected animals and control rats
264 was also compared.

265 It was found that the proportion of ChAT-positive neurons was similar in the
266 submucous as well as in the myenteric plexus of infected rats and control rats (Table
267 1).

268

269 *CALB-immunoreactive populations*

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

278

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

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

291

292 During course of infection, the proportion of CALB-immunoreactive neurons did not
293 change. However, we found a significant higher proportion of CALB-positive neurons
294 in the myenteric plexus of BDV-infected rats as compared to control rats (Table 1). In
295 the submucous plexus, the relative number of CALB-immunoreactive neurons was
296 similar between infected rats and control rats (Table 1).

297

298 **Discussion**

299 This study showed that after intracerebral infection of Lewis-rats, BDV-
300 immunoreactivity can be found in neurons of the enteric nervous system (ENS).
301 Within the ENS, submucous neurons became BDV-immunoreactive to a higher
302 extent than myenteric neurons. In addition, BDV seemed to replicate in specific
303 subpopulations of neurons which express immunoreactivity for ChAT and CALB, and

304 the relative number of CALB-immunoreactive neurons was higher in the myenteric
305 plexus of infected animals as compared to non-infected.

306

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

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

321 The gastrointestinal tract is connected to higher centres via the parasympathetic and
322 sympathetic nervous system and via spinal afferent neurons (Brookes and Costa,
323 2006). In the proximal colon, the prominent extrinsic input originates from
324 sympathetic fibres (Brookes and Costa, 2006). Sympathetic ganglia as well as dorsal
325 root ganglia have been found to be a route for BDV spread after intracerebral
326 infection (Carbone et al., 1987). Although extrinsic colonic innervation was not
327 investigated in the present study, it seems likely that the virus entered the enteric
328 nervous system via sympathetic neurons or via spinal afferent fibres.

329 Sympathetic fibres as well as spinal afferents terminate in the myenteric plexus as
330 well as in submucous ganglia (Patterson et al., 2003; Brookes and Costa, 2006). This
331 is in accordance with the observed appearance of BDV-antigen in both submucous
332 and myenteric neurons at similar points in time p.i.. However, no data are available at
333 present regarding the extent of projection of sympathetic fibres and spinal afferents
334 to submucous and myenteric ganglia. Consequently, it is possible that the higher
335 amount of BDV-antigens in the submucous than in the myenteric plexus is due to a
336 denser extrinsic innervation of submucous neurons. However, supporting data for
337 this hypothesis are still lacking.

338 Besides the centripetal BDV transmission via the olfactory nerve to the CNS (Hornig
339 et al., 2003) and subsequent centrifugal spread, an oronasal infection may also
340 directly target enteric neurons projecting to the gastrointestinal epithelium. Presence
341 of BDV in the gastrointestinal tract can be presumed, since in persistently infected
342 animals BDV can be found in the feces (Sierra-Honigmann et al., 1993). If the
343 assumption is true that BDV-infection enters the body via the enteric nervous system,
344 virus replication in neurons innervating the epithelium, i.e. submucous neurons is
345 likely. However, so far no data are available concerning the susceptibility of
346 submucous and myenteric neurons to BDV replication. However, Carbone et al.
347 (1987) could not demonstrate an oral BDV-infection with subsequent virus
348 transmission via the enteric nervous system.

349

350 In our study, cholinergic subpopulations were the primary target for BDV replication.
351 This finding is consistent with studies obtained in the CNS of rats, where the
352 cholinergic system is also a main target of BDV infection (Gies et al., 2001). As a
353 consequence, the number of cholinergic neurons in the CNS decreases during BDV
354 infection (Gies et al., 2001). These changes cannot be found in the cholinergic

355 system of the ENS. In our study, the number of submucous and myenteric neurons
356 as well as the proportion of ChAT-positive neurons remained unchanged during BDV
357 infection. Therefore, it seems unlikely that BDV induces gastrointestinal dysfunctions
358 by disturbing some enteric neuronal subpopulations in Lewis rats. To date,
359 gastrointestinal dysfunctions during BDV-infection have been described only in
360 horses (Bode et al., 1994; Ludwig and Bode, 2000; Richt et al., 2000), suggesting
361 species differences of BDV-induced morphological alteration of subpopulations in the
362 ENS. Further studies are required to test whether the function of enteric neurons is
363 altered during BDV-infection in horses.

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

369 In the myenteric plexus of BDV-infected rats, 29% of neurons expressed
370 immunoreactivity for CALB. In control rats the portion of CALB-immunoreactive
371 neurons was only 15%. Such an alteration could not be detected in the submucous
372 plexus. However, regarding the submucous plexus it must be emphasized that about
373 84% of submucous neurons expressed CALB-immunoreactivity in control rats. An
374 upregulation of CALB-immunoreactivity would therefore possibly be masked by inter-
375 individual variances in the proportion of CALB-positive neurons.

376 It remains unclear if CALB-expression was generally upregulated in myenteric
377 neurons of BDV-infected rats, or if upregulation was restricted to neurons which were
378 directly infected by BDV. Analyzing the proportion of CALB-positive neurons in
379 myenteric subpopulations of infected rats, we found 15% of myenteric neurons to be
380 BDV-negative and CALB positive. This relative number (15%) of CALB-positive

381 neurons can be also found in the myenteric plexus of control rats. Therefore it seems
382 likely that BDV does not specifically infect CALB-positive neurons, but BDV-infection
383 induces the expression of CALB. In this regard, function of CALB is still not clear.
384 However, recent data indicate that CALB plays a major role in different cell types in
385 protecting against apoptotic cell death (Christakos et al., 2003). Apoptosis of central
386 neurons is also caused by BDV-infection in neonatally infected rats (Hornig et al.
387 1999, Weissenbock et al. 2000). Consequently, the upregulation of CALB-expression
388 in the ENS might be a protective mechanism to prevent neurons from cell death.

389

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

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475 **Figure captions**

476

477 **Figure 1**

478 Photomicrographs showing the presence of BDV-specific antigen in the submucous
479 (**A,B,C,D**) and myenteric (**E,F**) plexus. **A, B** In the majority of submucous ganglia,
480 only nerve fibres (indicated by an arrow) are BDV immunoreactive 4 weeks p.i.. After
481 14 weeks p.i., BDV-immunoreactive somata (examples are indicated by arrows) can
482 be found in the submucous (**C, D**) and myenteric (**E, F**) plexus.

483

484 **Figure 2**

485 Proportion of BDV-positive neurons in the submucous (open circles) and myenteric
486 (filled circles) plexus of infected animals. Results were calculated from analysis of
487 10062 myenteric and 4209 submucous neurones from 24 myenteric and 24
488 submucous plexus preparations of 12 animals. The preparations were stained using
489 antibodies against the general neuronal marker NSE and against BDV. Relative
490 number of submucous neurons infected with BDV increased up to 6 weeks p.i. and
491 tended ($p=0.075$) to further increase up to 10 weeks p.i.. Proportion of BDV-positive
492 myenteric neurons did not differ significantly between the different infection times.
493 Asterisks indicate significant differences ($p<0.05$, Student's-t-test) between the
494 submucous and myenteric plexus. a,b: different letters indicate significant differences
495 ($p<0.05$, one way-ANOVA with subsequent Student-Newman-Keuls test) in the
496 submucous plexus between different infection times.

497

498 **Figure 3**

499 Photomicrographs showing a submucous (**A, B**) and a myenteric (**C, D**) ganglion 6
500 and 4 weeks p.i., respectively. Tissues were double labelled against ChAT (**A, C**) and

501 BDV (**B, D**).The vast majority of BDV-positive neurons express immunoreactivity for
502 ChAT (examples are indicated by arrows).

503

504 **Figure 4**

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

509

510 **Figure 5**

511 Relative amount of subpopulations in the submucous (**A, B**) and myenteric plexus (**C**,
512 **D**) of BDV-infected rats. Data from 9 (6-14 weeks p.i.) and 12 (4-14 weeks p.i.) rats
513 are pooled for the submucous and myenteric plexus, respectively. The bar charts **A**
514 and **C** show subpopulations revealed by staining against NSE, BDV and ChAT.

515 Analysis of immunoreactivity for NSE, BDV and CALB revealed subpopulations
516 shown in the bar charts **B** and **D**.

517 A, B,C,D: Columns within each graph are significantly different ($p < 0.05$, one way
518 ANOVA with subsequent Student-Newman-Keuls test) if they do not share a
519 common letter; no significant differences were found between the subpopulations
520 shown in **A**.

Figure 1

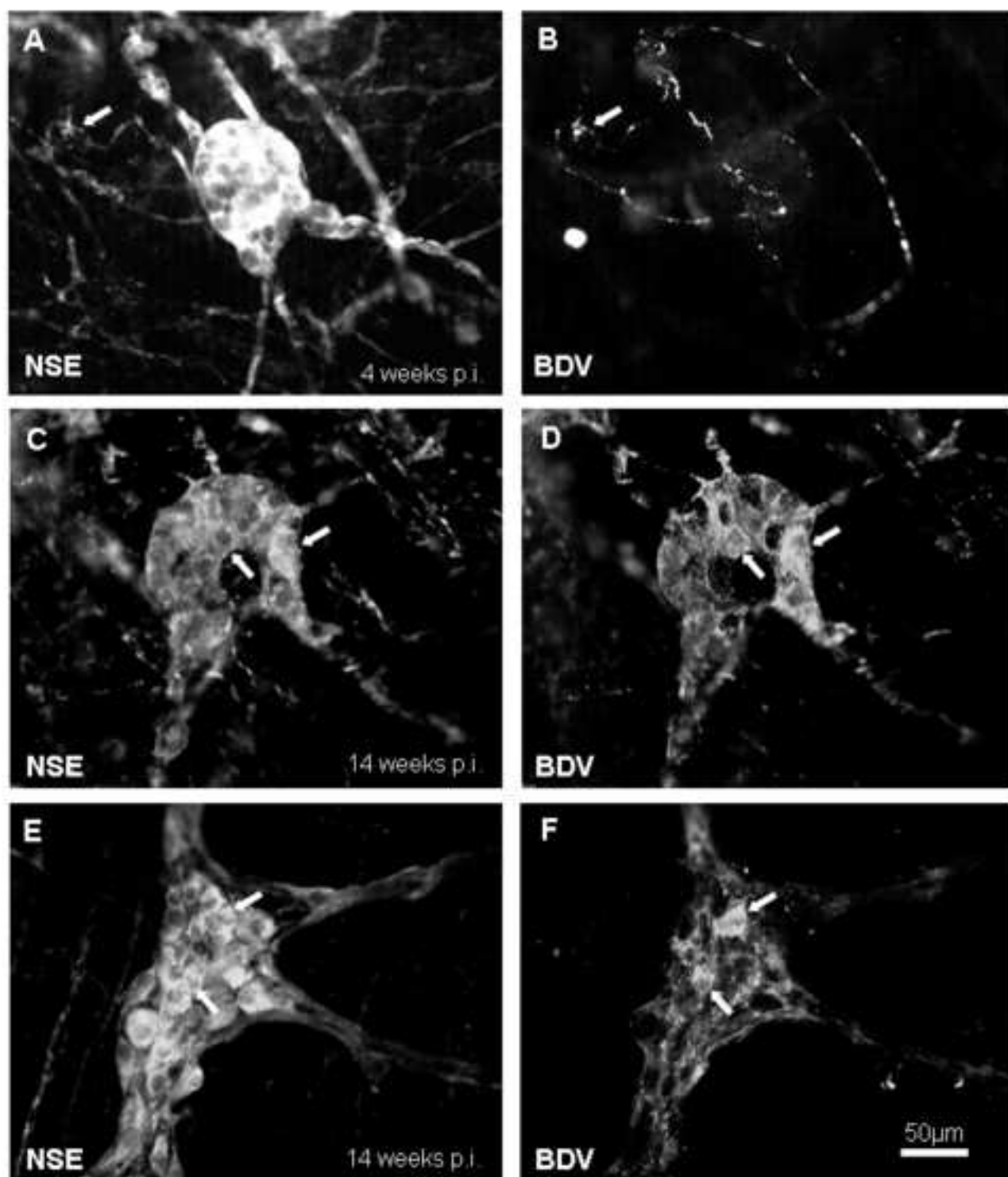


Figure 2

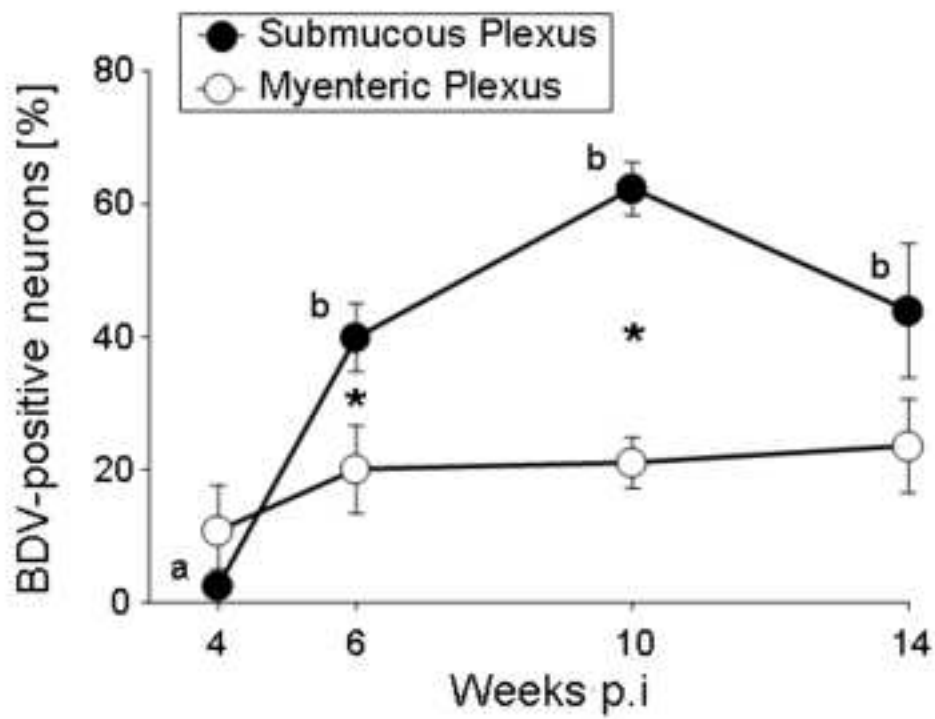


Figure 3

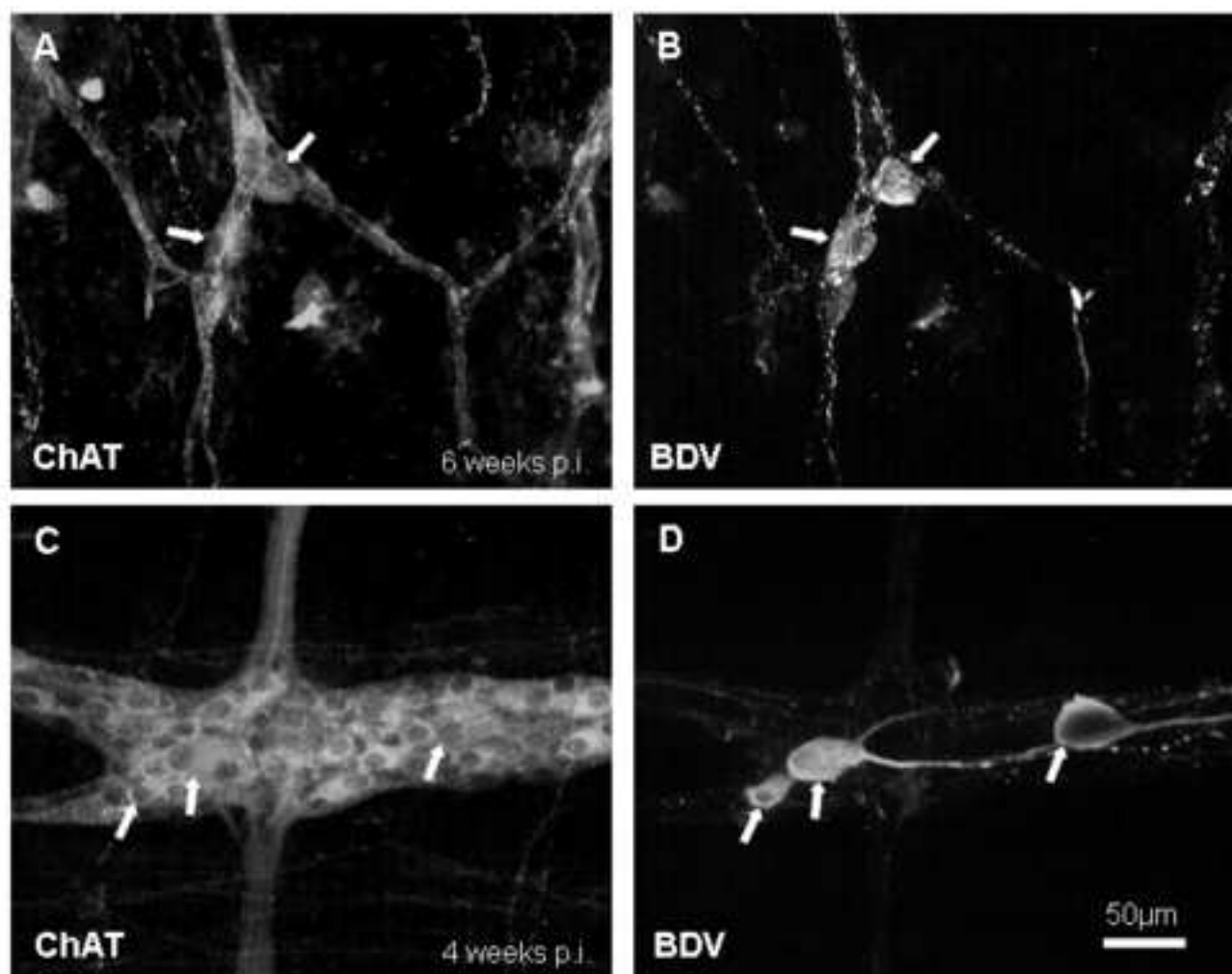


Figure 4

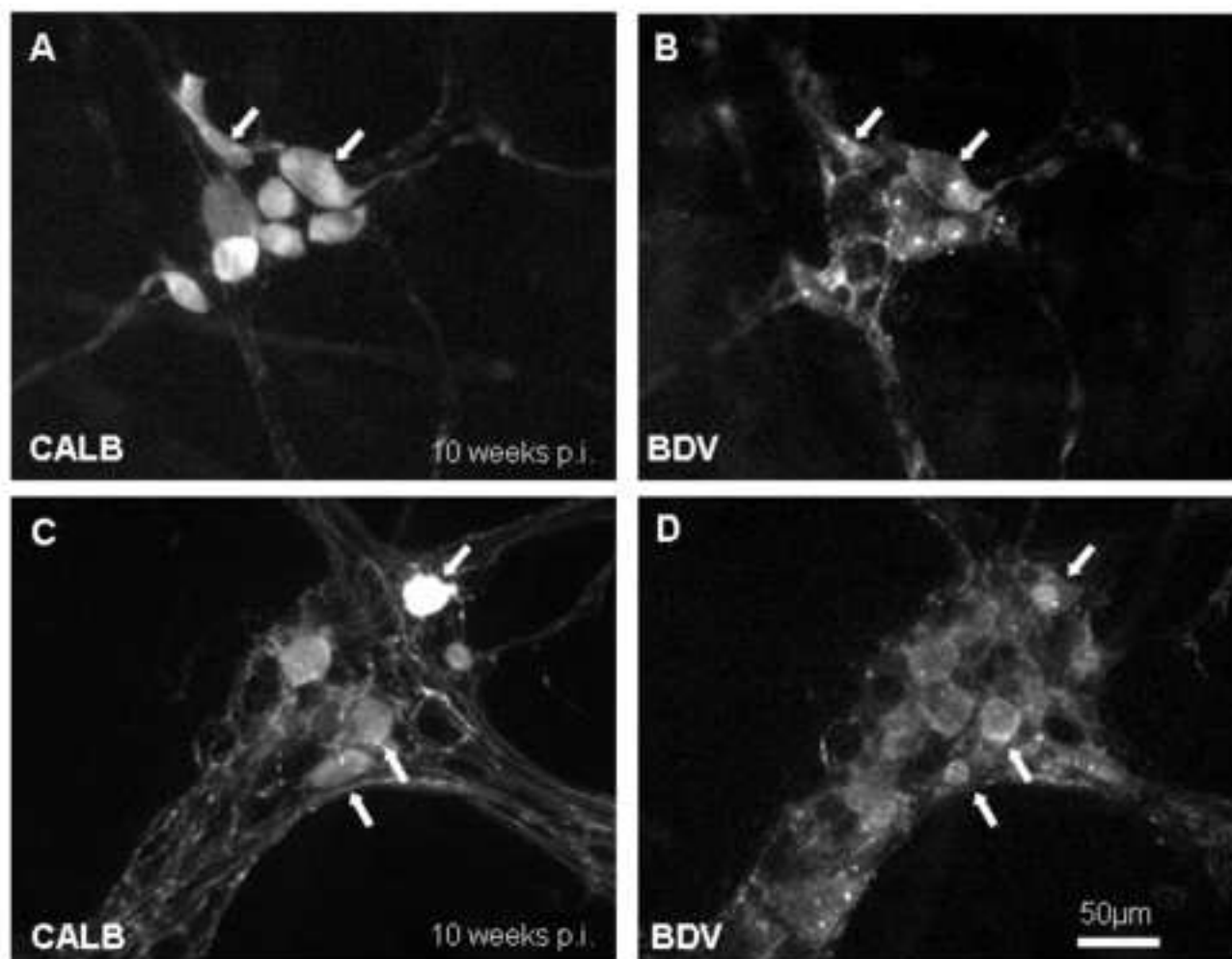
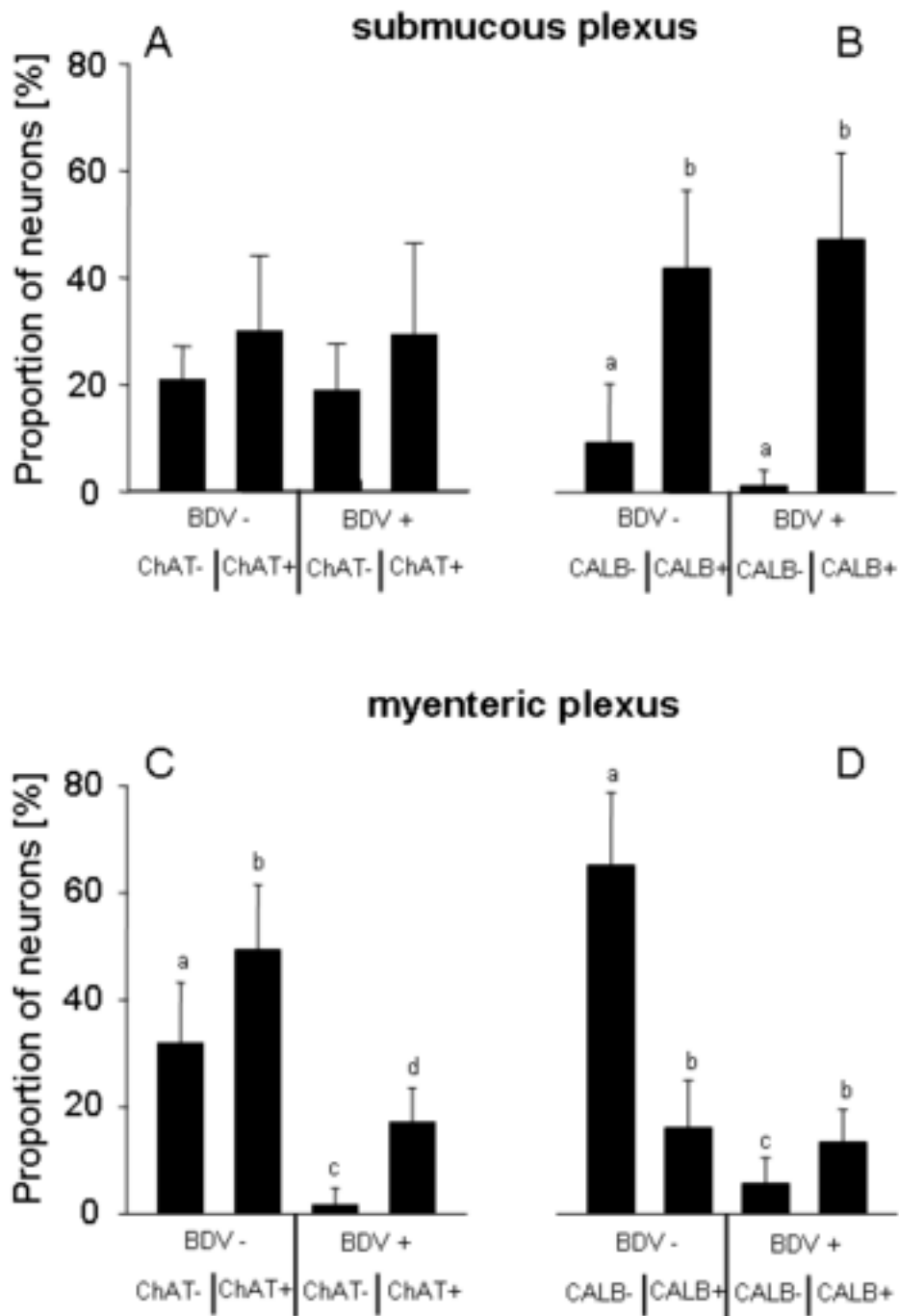


Figure 5



	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

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

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.

	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

Table 2**Immunoreactivity for BDV in ChAT-positive and CALB-positive neurones**

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.