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Cortical cholinergic decline parallels the progression of Borna virus encephalitis

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Borna disease virus (BDV)-induced meningoencephalitis is associated with the dysfunction of the cholinergic system. Temporal development of this cholinergic decline during pre-encephalitic and encephalitic stages of BDV infection remains however elusive. Changes in choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities were therefore determined in the cerebral cortex, hippocampus, striatum, amygdala and cholinergic basal forebrain nuclei (ChBFN) of rats infected with BDV. Immunocytochemistry for ChAT and vesicular acetylcholine transporter (VAChT) was employed to identify morphological consequences of BDV infection on cholinergic neurons. Whereas both ChAT and AChE activities changed only slightly under pre-encephalitic conditions, the encephalitic stage was characterized by a significant decrease of ChAT activity in the cerebral cortex, horizontal diagonal band of Broca (hDBB), hippocampus and amygdala concomitant with a marked reduction of AChE activity in the cerebral cortex, hDBB and hippocampus. The striatum and medial septum remained unaffected. ChAT and VAChT immunocytochemistry revealed prominent axonal degeneration in affected cortical and limbic projection areas of ChBFN. In summary, our data indicate progressive deterioration of forebrain cholinergic systems that parallels the progression of BDV encephalitis. *NeuroReport* 12:3767–3772 © 2001 Lippincott Williams & Wilkins.

Key words: Borna disease virus; Cholinergic system; Encephalitis; Inflammation; Neurodegeneration

INTRODUCTION

Infection with the Borna disease virus (BDV) leads to meningoencephalitis characterized by a severe lymphocyte-mediated immunopathological reaction of the host organism [1]. BDV is a non-cytolytic neurotropic non-segmented negative-stranded RNA virus that predominantly infects horses under natural conditions. Experimentally induced BDV infection, however, may lead to encephalitis in a broad spectrum of host species ranging from birds to rodents and non-human primates [2]. Serological data and detection of viral RNA indicate the possibility of viral transmission to humans, and BDV has been implicated in the development of psychiatric disorders, such as schizophrenia [3] and depression [4]. Under experimental conditions, Lewis rats infected with the highly neurotropic BDV develop a biphasic illness. The first phase is characterized by hyperactivity, aggression and loss of bodyweight, while apathy and paralysis predominate in the terminal phase.

Cholinergic neurons localized in basal forebrain nuclei, namely the magnocellular nucleus basalis, the vertical/horizontal diagonal bands of Broca (v/hDBB) and the medial septum (MS), project to the cerebral cortex and limbic structures, such as the hippocampus and amygdala [5,6]. It is worth noting that intrinsic cholinergic interneurons are present in many of these projection areas. From a functional point of view, ascending cholinergic projections modulate the integration of sensory and motor information, learning and memory processing [7], whereas intracortical and intra-hippocampal cholinergic interneurons may modulate oscillatory states of the cerebral cortex and hippocampus [8]. The cholinergic system also serves as an interface between the central and peripheral parts of the immune system and influences immune responses to a variety of stimuli [9].

Previous neuroanatomical studies in our laboratories [10] revealed changes in the cholinergic system, in particular a decrement of the acetylcholine-synthesizing enzyme choline acetyltransferase (ChAT, EC 3.2.1.6), in the latent (also termed pre-encephalitic) stage of BDV infection. Interestingly, the morphological changes occurred either concomitant with or even preceding the T-lymphocyte infiltration of the CNS that is a hallmark of the onset of encephalitis [1]. Considering the importance of the cholinergic system in various neural mechanisms and its sensitivity to BDV infection [10], in the present study we

investigated whether the BDV-induced cholinergic decline parallels the disease progression. We therefore biochemically assayed ChAT and acetylcholinesterase (AChE, EC 3.1.1.7) activities in the cerebral cortex, striatum, basal forebrain cholinergic nuclei, hippocampus and amygdala both under pre-encephalitic and clinically developed encephalitic conditions.

We also provided morphological correlates of the biochemical findings by means of immunocytochemistry for ChAT and the vesicular acetylcholine transporter (VAChT).

MATERIALS AND METHODS

Viral infection and experimental design: Adult Lewis rats were infected by injection of infectious brain homogenate (50 µl; Giessen strain He/80, 6×10^3 $tcid_{50}$) in the left striatum and fronto-parietal cortex. The Giessen virus strain originates from the brain homogenate of a horse with natural Borna disease. Subsequently, viruses were passaged twice in rabbit brain, in mDCK cells and thereafter twice in newborn rat brains [10]. Experimental procedures were in accordance with the regulations of the NIH Principles of Laboratory Animal Care (1985). All efforts were made to minimize the number of animals and their suffering throughout the studies. The animals infected with BDV and showing no major signs of encephalitis were sacrificed 10 days after inoculation and served as the pre-encephalitic group ($n_{PE} = 16$). On the other hand, all rats with a survival period of 14–22 days showed characteristic signs of encephalitis ($n_E = 25$). Our criterion in the group of encephalitic animals was the appearance of apathy and paralysis of the hindpaws. Control animals received injections of normal brain homogenate (50 µl, $n_c = 15$) and were sacrificed after a survival period of 16 days. Subsequently, the animal groups were further sub-divided for biochemical ($n_{PE} = 9$, $n_E = 14$, $n_c = 8$) and histochemical ($n_{PE} = 7$, $n_E = 11$, $n_c = 7$) measurements.

Tissue preparation and biochemical measurements: For biochemical analysis the animals were deeply anesthetized with 6% sodium-pentobarbital (i.p.). Thereafter, rats were decapitated and their brains were quickly removed. Brain samples were immediately frozen at −30°C for 10 min and then stored at −80°C until further processing. Brain regions of interest were dissected according to the micro-dissection procedure of Palkovits and Brownstein [11]. In brief, coronal brain sections were cut on a cryostat microtome at a thickness of 300 µm. Subsequently, samples of the cingulate, frontal and parietal cortices, striatum, MS, hDBB, hippocampus and amygdala were punched out under light microscopy control with a punch needle 2 mm in diameter. Samples of identical regions of the two hemispheres were then pooled in each animal. Occasionally, corresponding samples (e.g. MS, hDBB) from two animals were also pooled to reach a minimum tissue weight necessary for biochemical measurements. ChAT activity was measured by the radiochemical method of Fonnum [12]. Final concentrations in the incubation mixture were: 0.6 mM [^{14}C]acetyl-coenzyme A (sp. act. 60 mCi/mmol; Amersham Pharmacia Biotech., Roosendaal, The Netherlands), 300 mM NaCl, 50 mM phosphate buffer (pH 7.4), 10 mM choline-HCl, 20 mM EDTA and 0.1 mM physostigmine sulfate (all chemicals were purchased from Sigma, St. Louis, MO, USA). The radioactivities of the samples were measured in a liquid scintillation counter. ChAT activity was expressed as nmol of ACh synthesized/h/mg protein. AChE activity was determined by means of spectrophotometry at 412 nm, as initially described by Ellman *et al*. [13], where pseudocholinesterase activity was inhibited by tetraisopropyl pyrophosphoramide (iso-OMPA, Sigma). AChE activity was expressed as nmol ACh hydrolyzed/min/mg protein. The protein contents of the samples were measured by means of a spectrophotometric method at 650 nm according to Lowry *et al*. [14] using Folin phenol reagent and bovine serum albumin as standard.

Perfusion and immunocytochemistry: Under deep sodium pentobarbital anesthesia (i.p.) the rats were perfused transcardially with 500 ml fixative containing 4% paraformaldehyde, 0.2% picric acid and 0.05% glutaraldehyde, preceded by a short pre-rinse with physiological saline (30 ml). Subsequently, whole brains were removed and post-fixed in the same fixative for 2 h. Brains were cryoprotected by overnight storage in 25% sucrose buffered with 0.1 M phosphate-buffered saline (pH 7.4). Coronal sections were then cut at 50 µm on a freezing microtome. For ChAT and VAChT immunocytochemistry, free-floating sections were rinsed several times in Tris–HCl buffered saline (TBS, 0.05 M, pH 7.6; Chemicon International Ltd, Harrow, UK) and incubated in 10% normal horse or rabbit serum, respectively, for 30 min at room temperature. The primary antibodies, mouse-anti ChAT (4 µg/ml; [15]) and goat anti-VAChT (1:2000; Phoenix Pharmaceuticals, Mountain View, CA, USA; [16]), were diluted in TBS to which 0.5% Triton X-100 had been added in order to enhance the penetration of the antibodies and incubated at 4°C for 48 h. After thorough rinsing in TBS, the sections were reacted with biotinylated horse anti-mouse IgG or rabbit anti-goat IgG (1:100, Vector, Burlingame, CA, USA), respectively, for 2 h at room temperature. Following rinsing in TBS the sections were incubated in ABC solution (Vectastain ABC Elite Kit, 1:200, Vector) for 2 h at room temperature. Tissue-bound peroxidase was visualized using 3,3′-diaminobenzidine (DAB, Sigma) as chromogen with nickel enhancement (in Tris–HCl buffer, 0.05 M, pH 8.1) and H_2O_2. All steps were performed under continuous gentle agitation of the sections in the solutions. Omission of the primary antibodies yielded no detectable immunolabeling (data not shown).

Statistics: Effects of BDV infection on ChAT and AChE activities were statistically evaluated using one-way ANOVA (SPSS for Windows version 10.1.1, SPSS Inc., Chicago, IL, USA). If any statistically significant change was found, pair-wise comparisons were performed using Tukey's *post-hoc* test. $p < 0.05$ was taken indicative of statistical significance. Data on enzyme activities were expressed as means ± s.e.m.

RESULTS

Significant effects of BDV infection were demonstrated on ChAT activity in the cingulate ($F(2,17) = 18.62$, $p < 0.01$), frontal ($F(2,20) = 8.09$, $p < 0.01$) and parietal cortices ($F(2,20) = 8.66$, $p < 0.01$), as well as hDBB ($F(2,12) = 12.82$, $p < 0.01$), hippocampus ($F(2,21) = 7.20$, $p < 0.01$) and amygdala ($F(2,16) = 4.94$, $p < 0.05$) (Table 1). AChE activity

Table 1. Effects of BDV encephalitis on choline acetyltransferase and acetylcholinesterase activities in the rat cerebral cortex, basal forebrain, and limbic system.

	Cingulate cortex	Frontal cortex	Parietal cortex	Striatum	MS	hDBB	Hippocampus	Amygdala
ChAT								
Control	37.13 ± 1.85	30.72 ± 1.02	29.56 ± 1.24	125.81 ± 5.99	63.53 ± 9.25	145.80 ± 20.61	38.77 ± 2.62	83.49 ± 7.12
Pre-encephalitis	35.12 ± 3.05	27.95 ± 1.22	28.57 ± 1.43	133.77 ± 8.29	54.30 ± 3.05	156.51 ± 4.07	34.44 ± 1.56	88.92 ± 8.82
Encephalitis	23.16 ± 1.38[a,b]	22.68 ± 1.93[a,d]	23.00 ± 1.01[a,d]	118.03 ± 9.96	46.35 ± 5.53	75.15 ± 5.09[a,b]	23.66 ± 3.99[a,d]	59.21 ± 5.99[a,e]
AChE								
Control	38.87 ± 5.99	36.05 ± 0.79	40.32 ± 1.98	239.70 ± 13.1	79.30 ± 15.1	227.50 ± 31.4	56.56 ± 2.07	66.60 ± 12.0
Pre-encephalitis	44.68 ± 3.13	30.05 ± 2.89	35.31 ± 2.30	214.40 ± 18.1	78.19 ± 9.5	227.10 ± 37.9	50.38 ± 3.93	89.20 ± 19.4
Encephalitis	29.76 ± 3.25[d]	28.77 ± 2.95	29.83 ± 1.62[a]	201.40 ± 18.5	69.90 ± 12.1	107.40 ± 23.5[c,d]	43.40 ± 6.26	69.23 ± 7.58

The development of BDV encephalitis leads to stage-specific alterations in both choline-acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities in various areas of the rat forebrain. Note the primary involvement of cerebral cortical regions, whereas the striatum and medial septum remained largely unaffected. Nine rats in the pre-encephalitic stage, 14 rats exhibiting characteristic signs of encephalitis and eight sham injected animals were used in the biochemical assays.

Data on ChAT and AChE activities were expressed as nmol/h/mg protein and nmol/min/mg protein, respectively. Statistical analysis was performed by one-way ANOVA followed by Tukey's *post-hoc* test where appropriate. [a] $p < 0.01$, [c] $p < 0.05$, [e] $p = 0.072$ *vs* control; [b] $p < 0.01$, [d] $p < 0.05$ *vs* the pre-encephalitic group. No statistical differences were found between the sham-infected and pre-encephalitis groups. Data were expressed as means ± s.e.m.

MS: medial septum; hDBB: horizontal diagonal band of Broca.

became significantly affected in the cingulate ($F(2,17) = 3.48$, $p < 0.05$) and parietal ($F(1,20) = 7.50$, $p < 0.01$) cortices, and hDBB ($F(2,12) = 5.81$, $p < 0.02$). *Post-hoc* analyses revealed only slight changes of both enzyme activities in the pre-encephalitis group compared with controls. In particular, ChAT activity exhibited a non-significant decrease in the cingulate, frontal and parietal cortical areas, MS and hippocampus, whereas it was slightly elevated in the striatum, hDBB and amygdala relative to the control group. Similarly, reduced AChE activity was measured in the frontal and parietal cortices, striatum, MS, hDBB and hippocampus, while increased enzyme activities were recorded in the cingulate cortex and amygdala, as compared to control animals. In contrast, severe reduction of both ChAT and AChE activities paralleled the further development of encephalitis. A significant decline of ChAT activity was recorded in the cingulate ($p < 0.001$ *vs* both other groups examined), frontal ($p < 0.01$ *vs* control, $p < 0.05$ *vs* pre-encephalitis) and parietal cortices ($p < 0.01$ *vs* sham-injected, $p < 0.05$ *vs* the pre-encephalitis group), hDBB ($p < 0.01$ *vs* both other experimental groups), hippocampus ($p < 0.01$ *vs* control, $p < 0.05$ *vs* the pre-encephalitis group) and amygdala ($p = 0.072$ and $p < 0.05$ *vs* control and pre-encephalitis animals, respectively). On the other hand, only a marginal decrement of ChAT activity was demonstrated in the striatum and MS, compared with both the sham-treated and pre-encephalitis groups (Table 1). Furthermore, significantly diminished AChE activity was measured in cingulate ($p < 0.05$ *vs* the pre-encephalitis group) and parietal cortical samples ($p < 0.01$ *vs* the control group), and in the hDBB ($p < 0.05$ *vs* both control and pre-encephalitis groups), whereas it remained largely unaffected in the frontal cortex, striatum, MS, hippocampus and amygdala, relative to either control groups investigated.

ChAT and VAChT immunocytochemistry in both sham-infected and pre-encephalitis animals visualized all major morphological hallmarks of the basal forebrain cholinergic system, such as large multipolar projection neurons (Fig. 1a,c), and dense fiber projections to the cerebral cortex, amygdala and hippocampus (Fig. 2a,c,e). In accordance with previous studies [17], layers I–II and V of the cerebral cortex received the most prominent cholinergic innervation (Fig. 2a–d), whereas fusiform ChAT-positive but VAChT-negative intrinsic cortical cholinergic interneurons were scattered in the cortical mantle (Fig. 2a,b). In BDV-infected pre-encephalitic animals, however, single swollen cholinergic fibers were present in the major projection pathways (data not shown) [10]. In basal forebrain cholinergic nuclei of BDV-infected animals showing classical signs of encephalitis, an obvious loss of ChAT-ir projection fibers and, to a lesser extent of cholinergic neurons, became evident (Fig. 1b,d). A severe decline of the density of ChAT-ir and VAChT-ir projections to the frontal and parietal cortices was also demonstrated, while VAChT-ir projections innervating the cingulate cortex were relatively spared (Fig. 2b,d). The most striking damage to cholinergic, in particular to VAChT-ir, fibers was demonstrated in the dorsal hippocampus (Fig. 2e,f). Whereas cholinergic projection fibers, under control and pre-encephalitis conditions, are mainly found in the infra- and supra-pyramidal zones of the CA1 and CA3 sub-fields and in the infra- and supra-

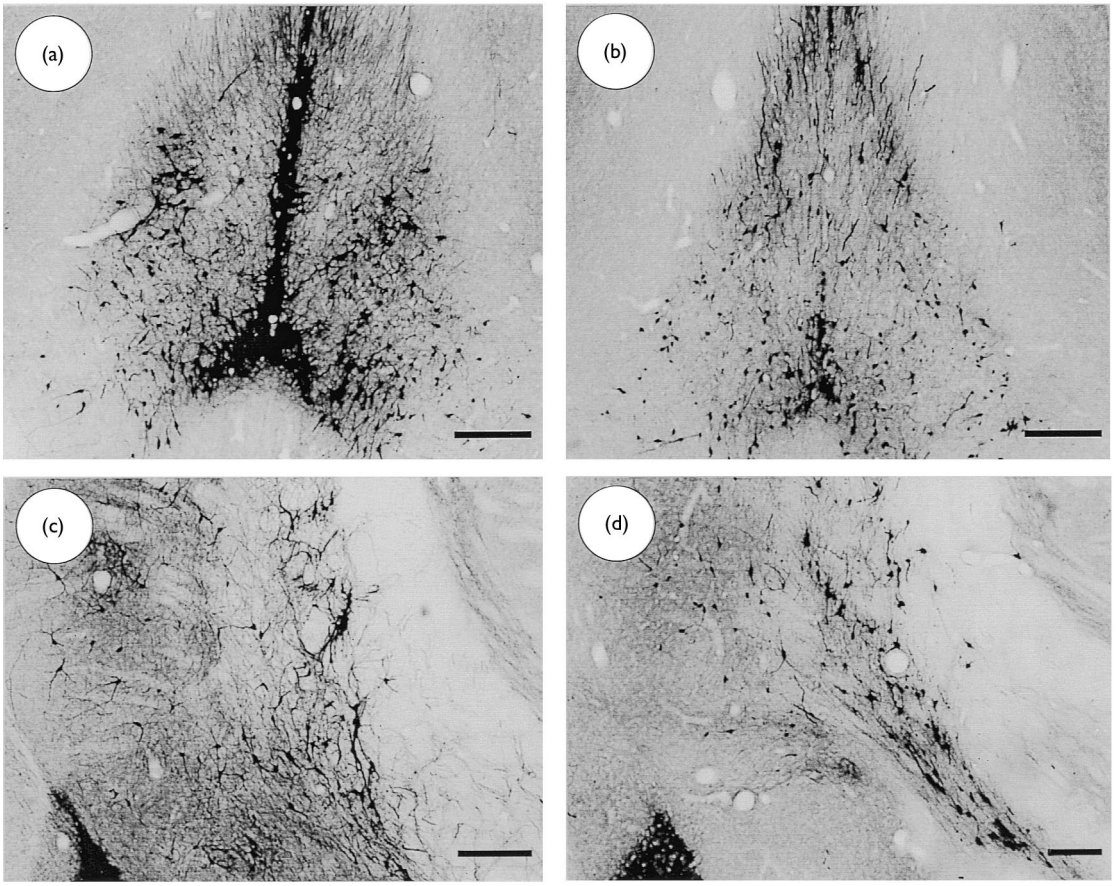

Fig. 1. Effects of BDV-induced encephalitis on choline acetyltransferase immunoreactivity in rat medial septum (**a**,**b**) and intermediate magnocellular nucleus basalis (**c**,**d**). A considerable decline of ChAT immunoreactivity became apparent in rats exhibiting characteristic signs of encephalitis (b,d) compared with control animals (a,c). Note the predominant loss of ChAT immunolabeling of fiber branches emanating from basal forebrain cholinergic projection neurons. Bars = 250 μm (a,b); 200 μm (c,d).

granular layers of the dentate gyrus (Fig. 2e), an almost complete loss of VAChT-ir fibers was observed in all layers during acute encephalitis (Fig. 2f). Residual cortical cholinergic fibers withstanding BDV infection were swollen with numerous bloated boutons (Fig. 2d,f). No remarkable differences were detected in the cholinergic innervation pattern of the striatum and amygdala (data not shown). The above pathological changes were observed in all animals exhibiting acute encephalitis. It is noteworthy, however, that more profound injury to cholinergic neurons was found in animals with advanced encephalitis (i.e. longer survival periods).

DISCUSSION

The present study provides neurochemical and morphological evidence on progressive cholinergic dysfunction following intracerebral injection of BDV that parallels the development of acute encephalitis. Our data demonstrate only a limited decline of ChAT and AChE activities in the pre-encephalitic stage, which became significant in rats exhibiting signs of encephalitis. A clear division of brain regions affected by BDV infection emerged. Whereas reduced enzyme activities were determined in the cerebral cortex, hDBB and hippocampus, the striatum, MS and amygdala remained largely unaffected.

Immunocytochemistry for ChAT and VAChT largely confirmed the biochemical findings, inasmuch as severe injury to the cortical and hippocampal fiber projections of basal forebrain cholinergic neurons was recorded in acute encephalitis. In some instances, however, the histochemical and biochemical data did not correlate (e.g. cingulate cortex); this might be due to a difference in the sensitivity of the different approaches.

Development of BDV-induced encephalitis is a bi-phasic process. Whereas early infection (pre-encephalitis) is accompanied by the infiltration of single lymphocytes in the brain, e.g. lymphocyte accumulation around blood vessels in the hippocampus [10] without remarkable behavioral changes, encephalitis is characterized by a massive neuroinflammatory reaction with mood changes and appearance of paralysis [1]. Since a restricted decrease of both ChAT and AChE activities was present in many brain areas investigated in the latent pre-encephalitic phase, it seems that pre-encephalitic conditions set the stage for the progressive cholinergic dysfunction in acute encephalitis. This hypothesis is supported by the present as well as earlier observations from our laboratory [10] indicating the presence of swollen and degenerating cholinergic axons already under pre-encephalitis conditions.

ChAT and AChE activities declined simultaneously both

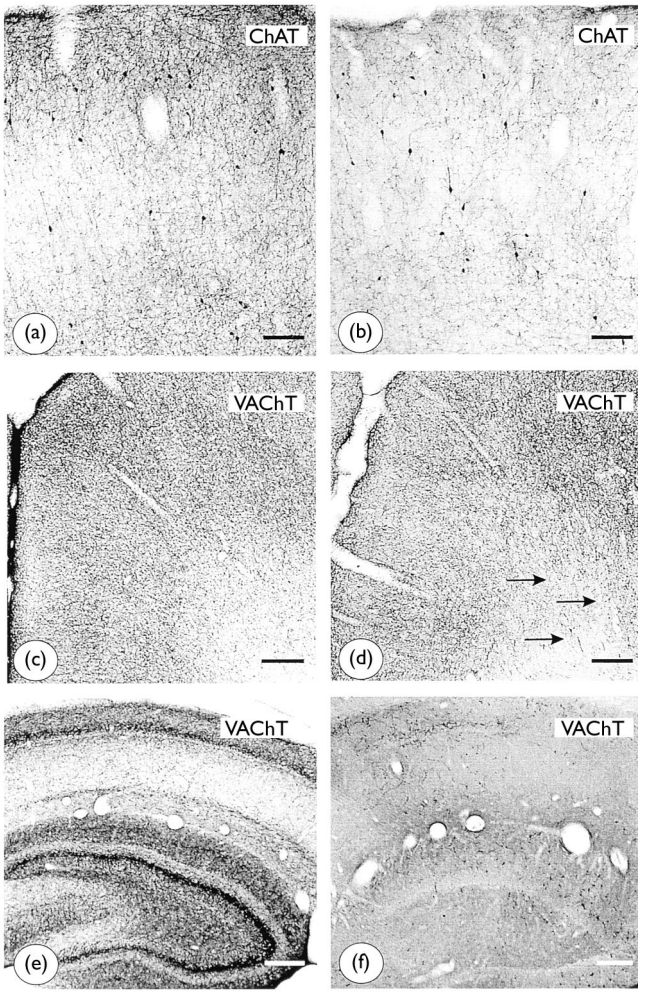

Fig. 2. BDV-induced loss of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) immunoreactivity in the cerebral cortex and hippocampus. ChAT-ir fiber innervation of the parietal cortex was considerably reduced in BDV-infected rats of the encephalitic stage (**b**) compared with control animals (**a**). Loss of cholinergic fibers was predominantly visualized in superficial layers of the cerebral cortex. Note the relative preservation of the innervation to the cingulate cortex (**d**) relative to sham-infected animals (**c**). Swollen, presumably degenerating, VAChT-ir axonal profiles (arrows) were observed in deep cortical layers in rats exhibiting BDV encephalitis (d), whereas these structures were absent in controls (c). In addition, a severe loss of VAChT-ir cholinergic fibers was evident in the hippocampus of rats showing signs of encephalitis (**f**) compared with the control group (**e**). Bar = 200 μm (c–f); 100 μm (a,b).

in pre-encephalitis and in acute encephalitis. In fact, a significant loss of ChAT activity was also demonstrated in the hippocampus, where AChE activity changed less extensively. Differences in the extent of changes for both enzymes may derive from the different cellular localization of these cholinergic markers. Whereas ChAT is exclusively located in cholinergic neurons and in their (pre-synaptic) terminals, AChE can be found in both cholinergic and cholinoceptive (post-synaptic) structures [18]. Hence, differential sensitivity of the cholinergic and cholinoceptive (e.g. glutamatergic, GABAergic) neurotransmitter systems to BDV infection, similar to other neurotoxic insults [19], might significantly affect the enzyme activities. Our studies, however, do not indicate whether BDV-induced damage to the forebrain cholinergic system is a primary or only a secondary event in the course of encephalitis. Further studies extending our knowledge on temporal changes of other neurotransmitter systems are therefore awaited to clarify their relation to the cholinergic breakdown.

Immunocytochemical analysis of ChAT -ir and VAChT-ir innervation of the cerebral cortex and hippocampus revealed that BDV encephalitis resulted primarily in axonal degeneration, whereas both basal forebrain cholinergic neurons and cortical interneurons remained relatively unaffected. These findings concur with those of Gonzalez-Dunia *et al*. [20], who showed significant synaptic loss as it was indicated by decreased expression of growth-associated protein 43 and synaptophysin. The predominant axonal pathology may also explain the relative preservation of ChAT and AChE activities in the MS. The frequent presence of beaded, presumably degenerating, varicosities in the MS may have interfered with the biochemical measurements, as ChAT might have accumulated in these structures. The possibility that the loss of ChAT activity in projection areas is partly due to the breakdown of axonal transport mechanisms cannot be excluded. Interestingly, no significant changes were found in enzyme activities in the striatum that might be linked to the presence of cholinergic interneurons, instead of projection neurons, in this structure.

Decreased cholinergic activity is not uniquely associated with BDV encephalitis but was also demonstrated in other viral infections, such as Venezuelan equine encephalomyelitis [21]. Moreover, cholinergic dysfunction was described in conditions accompanied by lymphocyte infiltration [22,23]. Taking these considerations together, its seems likely that acute neuroinflammation may lead to a severe damage of the cholinergic system and this cholinergic breakdown may then contribute to the development of behavioral abnormalities during acute encephalitis.

CONCLUSION

The present neurochemical and neuroanatomical data demonstrate progressive, end stage-related loss of ChAT and AChE activities in the cerebral cortex, hippocampus and hDBB during the development of BDV encephalitis. BDV infection predominantly elicited axonal degeneration, whereas basal forebrain cholinergic projection neurons as well as cortical and striatal cholinergic interneurons were relatively spared. From a functional point of view, the gradual decline of ChAT and AChE activities may be implicated directly in the development of behavioral dysfunctions associated with acute encephalitis. Although BDV infection has been implicated in the development of affective disorders [3,4], no causal relationship has been established between this infection and the psychiatric illnesses. Altered cholinergic functions are also associated with both schizophrenia [24] and depression [25]. Further clinicopathological studies are therefore necessary to reveal possible relationships between BDV infection, breakdown of the cholinergic system, and affective disorders.

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