Borna Disease Virus Replication in Organotypic Hippocampal Slice Cultures from Rats Results in Selective Damage of Dentate Granule Cells

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Borna disease virus (BDV) is a nonsegmented negative-strand RNA virus that persistently infects the central nervous system (CNS) and causes behavioral abnormalities in a broad range of vertebrates (17, 25). Depending on the age and immune status of the host, BDV infection may present as immune-mediated neurological disease with fatal outcome (Borna disease) or subtle behavioral alterations without overt inflammation (17, 25). BDV infection is potentially linked to psychiatric diseases, as BDV-specific antibodies were identified in sera of psychiatric patients with higher prevalence than in sera from control cohorts (24). However, attempts to confirm human BDV by nonserological methods, including detection of viral nucleic acid by nested reverse transcription-PCR or virus isolation, revealed inconsistent results (35); this issue is therefore still controversial.

Rats are the best-characterized animal models for studying BDV-induced pathogenesis. Depending on the age of the rat at the time of infection, the spectrum of BDV-caused diseases ranges from a progressive immune-mediated meningoencephalitis to behavioral abnormalities (17, 25, 26). In adult immunocompetent rats, BDV infection causes a biphasic disease characterized by a classical immune-mediated CNS disorder. This disease is associated with massive neuronal destruction and behavioral disturbances, the near-resolution of inflammatory infiltrates, virus persistence, and signs of chronic neurological disease. In contrast to infected adult rats, infection of neonatal Lewis rats results in lifelong persistence associated with behavioral abnormalities and a mild transient inflammation (18, 27–30, 37). These animals exhibit hyperactivity, cognitive defects, social behavior (play) abnormalities, and chronic anxiety. In infected newborn rats, BDV preferentially damages CNS areas that experience an extensive postnatal differentiation. One affected area is the dentate gyrus (DG) of the hippocampal formation, where granule cells degenerate following BDV infection (5, 18, 28). This brain structure plays a critical role in memory function, since the pyramidal CA3 cells of the hippocampus receive major excitatory input via the mossy fiber tract. The axons of the mossy fiber tract arise from granule cells in the DG. The DG, which itself receives input from various brain regions, forms a gateway for information transfer to the hippocampus. At birth, approximately 15% of dentate granule cells (DGCs) are formed, and the first mossy fiber terminals are observed on postnatal day 1 (P1) (11). However, the majority of granule cells are generated within the following 2 to 3 weeks (2, 31), and consequently a progressive development of mossy fiber projection and the maturation of synaptic structures appear during this postnatal period (1). This development reflects the temporal and topographical gradient of the neurogenesis and the positioning of granule cells in the developing DG. In addition, observations confirmed that neurogenesis of granule cells occurs in the DG of rodent brains throughout the life span (22). Cellular disorganization and neuronal death in the human hippocampus or DG are often associated with cognitive impairment and neurological disorders, as is...
known from patients suffering from Alzheimer’s disease or epilepsy (4, 12).

The selective loss of DGCs in infected newborn rats is most remarkable, because BDV is noncytolytic in cell culture, including primary neuronal cell cultures (13), as well as in the CNS of many hosts (36). Based on the observation that DGC degeneration occurs within the first weeks after birth, it was hypothesized that immature DGCs are highly vulnerable to BDV infection (25, 37). Additionally, BDV interference with the synaptic plasticity of DGCs could contribute to this pathology (13, 14). However, detailed studies of the early events of this neurodevelopmental pathology have been hampered by the lack of an accessible ex vivo culture system. Organotypic hippocampal slice cultures of newborn rats are widely used as an ex vivo system to study the postnatal development of neurons and synaptic connections under standardized conditions up to several weeks of age (15). Similar to in vivo development, the formation of the dentate gyrus and the maturation of granule cells occur within the first 2 to 3 weeks (38). We therefore tested whether neonatal rat hippocampal slice cultures could provide a suitable tool to study BDV-induced neurodevelopmental loss of DGCs in vitro. In such cultures, we observed selective damage of DGCs 21 to 28 days postinfection (p.i.) as indicated by a loss of DGC-specific calbindin staining, reduced number of mossy fiber boutons, and a retraction of mossy fiber axons. At these times after infection, almost all cells of the hippocampus, including pyramidal cells, were infected with BDV. In contrast, DGC formation occurred normally despite BDV infection until 14 days p.i., which indicates that the virus-induced loss of DGCs is triggered after the maturation of these cells. We therefore hypothesize that the DGC damage observed is a consequence of viral interference with mature granule cells rather than of the vulnerability of immature DGCs.

MATERIALS AND METHODS
Preparation of organotypic slice cultures. Hippocampi were dissected from rat pups (P0 to P2) and cut into 350-μm sections with a tissue chopper under sterile conditions. Hippocampal sections were transferred into petri dishes containing cold buffer solution of minimum essential medium supplemented with 2 mM glutamine at pH 7.3 and further cultivated on humidified porous membranes (Millicell Cell Culture Inserts CM30; Millipore Corporation) for various periods (14, 21, or 28 days) in slice culture medium as previously described (6). The medium was changed three times per week. Slice cultures were infected immediately after preparation with 1 to 1.5 μl of virus stock, corresponding to ca. 10 to 100 focus-forming units.

Immunohistochemistry. Cultures selected for immunofluorescence analysis were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 2 h. After several rinses with PB, the Millipore membrane with the cultures was cut off, mounted on an agar block, and resliced into 50-μm sections with a vibratome. Free-floating sections were then incubated in PB containing 5% normal goat serum and permeabilized with 0.1% Triton X-100 in PB for 30 min. Primary antibodies (anti-calbindin, 1:1,000, Chemicon; monoclonal antibody directed against the viral nucleoprotein [anti-Bo18], 1:50) (3) in phosphate-buffered saline (PBS) containing 1% normal goat serum were applied and incubated at 4°C overnight. After being washed with PBS, sections were incubated with a secondary antibody (Cy3-conjugated goat anti-rabbit immunoglobulin G [IgG], diluted 1:800, and Alexa 488-conjugated goat anti-mouse IgG, diluted 1:200, respectively) for 2 h at room temperature in the dark. For synaptophysin immunolabeling, cultures were processed as described above and incubated with anti-synaptophysin (diluted 1:2,000; Sigma) at 4°C overnight. As secondary antibody, Cy3-conjugated goat anti-mouse IgG was used (1:400). Sections were then washed with PBS, followed by staining with Hoechst nuclear stain. After being thoroughly rinsed in PB, slices were mounted onto gelatin-coated slides, embedded with immunomount (Shandon), and coverslipped. Sections were digitally photographed (Zeiss ApoTome).

Anterograde biocytin tracing. Prior to fixation, small crystals of the tracer biocytin (Sigma) were placed under visual control onto the dentate gyrus-hilus to label granule cells and their axonal mossy fiber projections. After incubation for 36 h, the cultures were fixed with 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% saturated picric acid in PB, pH 7.4, for 2 h. Thereafter, all cultures were resliced on a vibratome to 50-μm sections, permeabilized with 0.1% Triton X-100 for 30 min in PB, and incubated with ABC-Elite complex (1:50; Vector Laboratories) overnight at 4°C. Sections were developed using a nickel-cobalt-intensified 3,3’-diaminobenzidine protocol (34). Labeled sections were counterstained with cresyl violet, dehydrated, coverslipped with Entellan, and digitally photographed.

BDV virus stocks. Virus stocks from Oligo cells persistently infected with BDV strain He/80 were prepared as previously described (8) and subsequently diluted in PBS for 48 h. Determination of viral titers was carried out on Vero cells as previously described (8). Rat brain-derived virus stock strain RW98, (19) was obtained from Christian Sauder (Center for Biologies Evaluation and Research, U.S. Food and Drug Administration, Bethesda, Md.).

Western blot analysis. On average, eight individual slice culture sections were pooled and resuspended in Laemmli buffer (25), followed by ultrasonification. Protein extracts were size fractionated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride (Millipore) for Western blot analysis. The membrane was blocked in blocking solution (Gensios) supplemented with 0.05 g/ml saccharose and then incubated with either monospecific rabbit antibodies directed against N (8), calbindin (Chemicon), or a mouse anti-β-tubulin monoclonal antibody (clone TUB2.1; Sigma). After being washed, the blot was incubated with a 1:2,000 dilution of a peroxidase-conjugated donkey anti-mouse or anti-rabbit polyclonal antisera (Dianova) for 1 h at room temperature. Finally, bound enzymatic activity was detected by using the enhanced chemiluminescence system (ECL+) from Amersham.

RESULTS

Infection of hippocampal slice cultures from newborn rats with BDV results in the loss of calbindin-positive granule cells. To study BDV-induced neurodevelopmental damage of DGCs in vitro, we prepared hippocampal slice cultures from neonate (P0 to P2) Lewis rats. Untreated control hippocampal cultures showed a typical cellular arrangement of granule cells and a normal mossy fiber projection in the CA3 region of the hippocampus after 28 days of culturing as determined by immunofluorescence analysis using an antibody against calbindin, a marker protein for differentiated granule cells (Fig. 1A, top left) (19). This antibody also recognizes calbindin-positive interneurons in hippocampal subfields. A similar calbindin-staining pattern was observed after challenging the slice cultures with a mock-virus stock prepared from uninfected oligonucleotide cells (Fig. 1A, top right), which excluded the possibility that cell- or buffer-derived components interfered with normal hippocampal development. Infection of slice cultures with cell culture-derived BDV (strain He/80) at P1 led to a pronounced loss of calbindin-expressing DGCs 28 days p.i. (Fig. 1A, bottom left). Likewise, mossy fiber axons were virtually absent in these cultures. Infection of the slice culture with a rat brain-derived BDV virus stock (19) also resulted in severe loss of calbindin-positive DGCs (Fig. 1A, bottom right).

Western blot analysis using total cell extracts of these slice cultures confirmed that the calbindin levels were significantly lower in BDV-infected cultures than in uninfected cultures (Fig. 1B, middle). In addition, these analyses revealed that the slice cultures were thoroughly infected by BDV (Fig. 1B, top). Further immunofluorescence labeling using a monoclonal antibody (Bo18) directed against the viral nucleoprotein (3) showed almost complete infection of all cultivated neurons in...
the hippocampus, as well as the remaining calbindin-positive granule cells (Fig. 2A and D) in the dentate gyrus. Bo18 specifically recognized BDV, and no specific signals were observed in uninfected slice cultures (data not shown). The virus-infected cells showed the typical nuclear dot-like staining (Fig. 2B) that most likely represents the BDV replication sites (32). Several pyramidal neurons also showed prominent immunoreactivity in the dendrites and spines (Fig. 2C), reflecting accumulation of viral proteins throughout the entire neuronal cell.

**BDV-induced loss of calbindin-positive DGCs occurred immediately after formation of the dentate gyrus.** In untreated cultured slices, the cytoarchitecture of the dentate gyrus developed after neonate explantation, and a typical granule cell layer was formed (Fig. 1A). The calbindin-immunoreactive dendrites of the granule cells were distributed throughout the entire molecular layer, and the axons had grown out and invaded the hippocampal target field (Fig. 1A). These structural signs indicate normal neuronal differentiation and synapse formation within the first 2 weeks after neonate explantation. To investigate whether the BDV-induced loss of calbindin-expressing DGCs affects formation of the DGC layer, we analyzed BDV-infected slice cultures at 14 and 21 days p.i. At 2 weeks p.i., the cellular arrangement was comparable to that of untreated control cultures; no obvious loss of calbindin-positive granule cells and mossy fiber projections had occurred at that stage (compare Fig. 3A and Fig. 1), although almost all granule cells were BDV infected (Fig. 3E). Intriguingly, at 21 days p.i. only a few calbindin-positive DGCs and mossy fiber axons remained (Fig. 3B), and the organization of the DGC layer had dissolved (Fig. 3D), suggesting that the BDV-induced loss of calbindin-positive DGCs occurred after the granule cells integrated into the dentatohippocampal neuronal circuit.

**BDV infection results in reduced numbers of mossy fiber boutons and retraction of mossy fiber axons.** The observed lack of calbindin-positive mossy fiber projections in BDV-infected slice cultures might reflect a selective loss of granule cells and their processes or, alternatively, a down-regulation of calbindin expression in these neurons below the level detectable by immunostaining. To examine the distribution of mossy fiber synapses, infected and mock-infected slice cultures were analyzed 14 and 21 days p.i. by immunofluorescence for the presence of mossy fiber boutons, using antibodies directed against synaptophysin, a marker protein of synaptic vesicles (9). As indicated in Fig. 4A, the mossy fiber boutons, which contact the dendrites of the pyramidal cells in the CA3 region, differ in size from other synapses due to the formation of giant synapses and are thus easy to identify and to distinguish from other synapses. At 14 days p.i., there was no obvious difference in the number of mossy fiber boutons between mock-infected and BDV-infected slice cultures (Fig. 4B, left). In contrast, we observed a strong reduction in the numbers of mossy fiber boutons at 21 days p.i. compared to mock-infected slices (Fig. 4B, right).
The results shown in Fig. 4 suggest that BDV interferes with the maintenance of these synapses and might be related to axonal retraction. To further assess this possibility, we visualized the mossy fiber axon projections from 21-day p.i. cultures using anterograde tracing by injecting biocytin into the dentate gyrus. This tracing analysis was combined with Nissl staining to evaluate the cytoarchitectural organization. In both BDV-infected slices and in control cultures, the pyramidal cell layers were clearly identifiable; other neuronal cell types in the dentate gyrus and in the hippocampus were maintained and showed normal morphology (Fig. 5A, C, and E). Furthermore, in all cultures biocytin-labeled hilar neurons correctly projected above a Nissl stain-treated granule cell layer (Fig. 5E) or granule cell remnants (Fig. 5A and C). In mock-infected slice cultures, a well-developed granule cell layer was visible showing several biocytin-stained granule cell somata with dendrites (Fig. 5E) and mossy fiber axons running towards the CA3 region of the hippocampus. These axons formed many characteristic giant mossy fiber terminals in the target area (Fig. 5F). In contrast, in BDV-infected cultures only a few of the traced cultures showed mossy fiber axons with typical boutons (Fig. 5D), while others were devoid of any biocytin-labeled mossy fiber terminals (Fig. 5B). Accordingly, the granule layers of BDV-infected cultures varied in size, sometimes showing biocytin-stained granule cells (Fig. 5A and C). These results indicate that DGCs are selectively damaged after BDV infection. Furthermore, identification of cells in the granule cell layer of BDV-infected slices (21 days p.i.) that can be labeled by the terminal deoxynucleotidyl transferase dUTP-biotin nick-end labeling technique indicate that DGCs die by apoptosis (data not shown).

DISCUSSION

BDV infection of newborn rats is a well-established model system for studying virus-induced behavioral abnormalities. One feature of this model is the virus-induced degeneration of granule cells of the DG during the first weeks after birth. The selective loss of these cells is puzzling, since BDV is known to be noncytolytic in cell culture, including in primary neuronal cells (13). Therefore, detailed studies to unveil the molecular mechanisms of this pathogenesis are only possible in the organ and thus seem to be restricted to in vivo experiments. Here, we tested whether organotypic hippocampal slice cultures from newborn rats could provide a suitable system to study BDV-induced pathology in this brain area. We showed that infection of such slice cultures resulted in the selective loss of calbindin-positive granule cells at 21 to 28 days p.i., paralleled by the reduction in number of mossy fiber boutons, retraction of their mossy fiber axons, and death by apoptosis. Other neuronal cells of the hippocampus, including pyramidal cells or hilar cells, were not affected in their viability after viral infection. Thus, BDV infection of rat hippocampus slice cultures results in selective neuronal damage of DGCs as observed in cultures.
from infected newborn rats and is therefore a suitable model to study early events of this pathology ex vivo.

The upstream mechanisms that lead to DG degeneration are not understood. Since the vast majority of granule cells are generated after birth, it was hypothesized that the proliferating capacity of these neurons may be one explanation for their selective vulnerability to BDV infection (25, 37). However, we can clearly demonstrate that mature DGCs exhibiting apical dendrites and axons are affected and die by apoptosis. Furthermore, in BDV-infected slice cultures and in uninfected cultures, an obviously normal granule cell layer had developed, since only a minor portion of the granule cell layer was formed at tissue explantation. The granular layer remained intact until 14 days p.i., despite infection of almost all cells of the DG. We therefore speculate that the BDV-induced damage of the granule cells begins after these cells have differentiated dendritic processes and formed their efferent mossy fiber axons (Fig. 6). This is in line with the observation that infection of newborn rats at P15, where the formation of the hippocampus is almost completed, still resulted in the degeneration of the DG (28). Furthermore, this strongly argues against a high vulnerability of immature granule cells towards BDV infection, as previously claimed (25, 37). In contrast, the viability of these cells is not compromised, since immature DGCs immunolabeled with Prox 1 (a transcription factor specifically expressed in a subset of immature DGCs) (21) are still present in 3-week-old cultures despite infection with BDV (unpublished data). However, we cannot exclude the possibility that the viability of some immature granule cells is affected after BDV infection.
Similarly, the neogenesis of immature DGCs might also be affected by BDV infection. Impaired neogenesis could account for the failure to replace damaged mature DGCs by immature granule cells. Unfortunately, little is known about the numbers of granule cell progenitors that are available to replace damaged mature DGCs (7, 20). It is therefore unclear whether immature BDV-infected granule cells can compensate for the massive loss of mature DGCs.
Our findings obtained with the hippocampal slice culture system confirmed previous assumptions that DGCs are selectively damaged after BDV infection. Other cells including the hilar mossy cells and pyramidal neurons survived the infection without obvious signs of damage. The factors involved in this selective vulnerability of one specific cell type in the hippocampus remains to be shown. As suggested by others (25), soluble factors released by resident glia cells could contribute to DGC degeneration. Alternatively, the accumulation of certain BDV proteins could be toxic for these cells, especially since proteins of other viruses (e.g., human immunodeficiency virus gp120) are suggested to induce neuronal damage in the neonatal period (16). Furthermore, the axonal flow might be severely impaired in BDV-infected neurons (9, 10). This assumption is based on the observation that synaptophysin, a marker protein of synaptic vesicles, is sometimes found in cell bodies and in axonal spheroids in infected neurons. In BDV-infected slices, synaptophysin immunofluorescence produced punctate signals distributed throughout the neuropil. Large accumulations of synaptophysin immunoreactivity were confined to the hippocampal mossy fiber projections, representing the giant synapses of these axons. The number of these synaptic structures declines from 14 days p.i. onward. However, aberrant synaptophysin labeling in the cell bodies was not observed at any time point examined, supporting this idea of alteration in axonal flow, followed by neuronal damage. Damage of DGCs could, alternatively, originate from interference of BDV with proper synaptic functions that may ultimately lead to axonal deterioration, synaptic disconnection from target neurons, and finally death of the DGCs. This view is compatible with recent data indicating that BDV can indeed disturb synaptic plasticity by blocking BDNF-induced signaling events that regulate neuronal growth and also synaptic transmission (13, 39).

Studying early events in the BDV-induced DG degeneration in hippocampal slice cultures now offers unique possibilities to unveil the mechanisms leading to this pathology. In particular, early time points of infection between 14 and 21 days p.i., where retraction of mossy fiber axons are observed first, are of particular interest. With the help of the recently established reverse genetic system that allows the generation of BDV entirely from cDNA (33), it should be possible to unveil the viral determinants that contribute to this pathology.

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FIG. 6. Model of BDV-induced neurodevelopmental damage of hippocampal cells by altered neuronal connectivity of granule cells. (Left) During the first week after birth, only a few pyramidal (open triangles) and some other cells get infected with BDV (indicated by green dots). At this point in hippocampal development, the maturation of granule cells (open circles) is not complete, and the outgrowing mossy fiber axons still harbor growth cones at their termini (indicated by black dots). Only a few mossy fiber axons establish synaptic contacts with target neurons (large red dots). (Middle) After 2 weeks, despite strong infection of pyramidal cells, as well as most of granule cells, differentiation of the latter cells is almost complete and most mossy fiber boutons are formed. At this developmental stage, a functional connectivity has been established. (Right) During the following week, mossy fiber axons retract and granule cells undergo cell death (dashed open circles).


