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Borna disease virus-induced hippocampal dentate gyrus damage is associated with spatial learning and memory deficits

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ABSTRACT: In neonatally inoculated rats, Borna disease virus (BDV) leads to a persistent infection of the brain in the absence of an inflammatory response and is associated with neuroanatomic, developmental, physiologic, and behavioral abnormalities. One of the most dramatic sites of BDV-associated damage in the neonatal rat brain is the dentate gyrus, a neuroanatomic region believed to play a major role in spatial learning and memory. The absence of a generalized inflammatory response to neonatal BDV infection permits direct effects of viral damage to the dentate gyrus to be examined. In this report, neonatally BDV-infected rats at various stages of dentate gyrus degeneration were evaluated in the Morris water maze, a swimming test that assesses the rats' capacity to navigate by visual cues. Our data demonstrate progressive spatial learning and memory deficits in BDV-infected rats that coincided with a gradual decline in the estimated hippocampal dentate gyrus neuron density. © 1999 Elsevier Science Inc.

KEY WORDS: Virus, Rat, Behavior, Brain, Morris water maze.

INTRODUCTION

Many behavioral and psychiatric disorders of children such as autism, attention deficit disorder and learning disabilities may result from subtle brain damage during critical periods in brain development [17,30,37,53]. Such neuropsychological abnormalities may have a viral etiology because viral infections have been associated with both abnormal brain development [6,12,26] and psychiatric illnesses [3,13,24,38,49,55]. However, only in rare cases have distinct behavioral anomalies been linked to specific sites of virally induced brain damage (e.g., herpes simplex virus infection of the temporal lobe and personality changes in humans [55] or vesicular stomatitis virus infection of serotonergic neurons and motor and behavioral abnormalities in rats [39]). Because of the paucity of documented cases of virus-induced brain damage associated with distinct behavioral abnormalities, there continues to be significant controversy concerning whether viral pathogens can cause specific psychiatric illnesses [29,35,52,56,65]. In part, this controversy may be the outcome of the limited number of animal model systems available with which to study the pathogenesis of distinct cognitive and other behavioral abnormalities after developmental brain damage associated with perinatal virus infection.

Borna disease virus (BDV) infection has been associated with some forms of human psychiatric diseases such as schizophrenia and affective disorders [2,7,27,61]. Borna disease virus is an 8.9 kb negative strand RNA virus in a new class of viruses, Bornaviridae, in the Mononegavirales order [11,18,20]. In the Lewis rat model of BDV infection, neonatally inoculated rats develop a persistent infection and neuroanatomic and behavioral disease without generalized meningitis or encephalitis [15,31,43]. The absence of cellular inflammatory responses after BDV infection of the neonatal central nervous system (CNS) allows for these neuroanatomic and behavioral abnormalities to be studied without the complications of global nonspecific immunopathologic brain damage [5,15,23].

One of the first sites of BDV replication in the rat brain is in the hippocampal formation, preferentially replicating in neurons of the dentate gyrus (DG) and CA3 to CA4 region of the hippocampus proper [14,15]. Within a 2- to 3-month period after infection, BDV-infected DG neurons become pyknotic and die, and a prominent astrogliosis develops at the site of the destroyed DG [15].

The hippocampal formation, in particular the DG, is associated with spatial learning and memory [25,46,47,58,59], for example, the ability to navigate within an environment using a wide variety of distal and proximal cues. In view of the extraordinary BDV-induced damage to the DG and the relationship between the DG and spatial learning and memory, neonatally BDV-infected rats were tested in the Morris water maze (MWM), which measures the rats' ability to use visual cues to navigate to a hidden platform [40]. Because of the progressive nature of the BDV-induced DG damage, assessed by estimating DG neuron density, performance in the MWM was serially evaluated as a function of time postinfection.

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MATERIALS AND METHODS

Rats

Two litters each of newborn Lewis rats (Harlan, Indianapolis, IN, USA) were inoculated intracranially either with 1×10^4 50% tissue culture infective doses of BDV-infected rat brain homogenate or with an equivalent volume of uninfected rat brain homogenate prepared as previously described [14]. After weaning, BDV-infected ($n = 13$) and uninfected ($n = 13$) rats were separated and housed individually. Rats were given food and water *ad libitum* and kept in fluorescent lighting with a diurnal cycle (14 h light, 10 h dark).

Three groups of naive uninfected and neonatally BDV-infected rats were tested in the MWM at 43 ($n = 10$), 53 ($n = 8$) or 72 ($n = 8$) days of age. The distribution of males and females was identical between the uninfected and BDV-infected groups at each time point. After testing, each group of uninfected and BDV-infected rats at each time point was killed for histologic evaluation of the brain. All rat experiments were conducted in accordance with the National Institutes of Health Guide regarding the care and use of animals.

Morris Water Maze

Spatial learning and memory were evaluated in the MWM paradigm [40]. The maze consisted of a circular plastic tank (110 cm in diameter, 50 cm high) filled with water to a depth of 25 cm and made opaque by the addition of Tempera pigment (Visual Systems, Baltimore, MD, USA). A plastic escape platform (100 cm^2) was submerged 1 cm below the water surface at a fixed location. The task required the rats to escape from the water by locating the hidden submerged platform. Testing was performed in an isolated room under fluorescent overhead lighting. Water temperature was maintained at 22–24°C, and air temperature was maintained at 28°C.

Maze Acquisition

On the day before testing, the rats were introduced to the MWM environment and trained to find the submerged platform as follows. Each rat was given two sets of six swims to become familiarized with the MWM and to reinforce the conditioned behavior of locating the platform as a means of escape (1 min between trials; 90 min between sets). After the familiarization period, a rectangular Plexiglas channel (60 × 25 cm) was placed in the MWM to direct the rats to the hidden platform. Rats were trained to locate the hidden platform by placing them on the platform for approximately 30 s and then placing them within the Plexiglas channel at increasing distances from the platform with each subsequent trial. Upon reaching the platform, rats were removed, dried and placed in a holding cage. Visual cues were absent during this training period.

Maze Testing

On the following day, the rats were tested for quantitative spatial learning and memory performance as indicated by navigation to the platform via visual cues (two-dimensional objects of different sizes, shapes and colors) placed on the walls of the maze tank. Rats were given a total of 20 trial swims, divided into four sets of five trials (1 min between trials, 90 min between sets). Trial swims originated from one of four randomly determined maze quadrants. Escape latency (swim time from insertion in tank to location of platform) was recorded on videotape. Rats were allowed 90 s to locate the platform during each trial. If the rat failed to reach the platform within 90 s, the rat was manually placed on the platform.

For each rat, mean trial escape latency was calculated by averaging the escape latencies recorded in each of the four sets of five trials. As the rats learn to navigate to the platform via visual cues, latency to the platform should decrease. Thus, percent improvement in maze performance, as a measurement of learning, was calculated by comparing the shortest mean escape latency of the four sets of five trials to the initial mean escape latency in the first set of trials for each rat.

Upon completion of the four sets of five trials, a probe trial swim was performed to test spatial memory. For the probe trial test, the escape platform coordinates were recorded and the platform was removed from the maze. A designated 12.5-cm diameter probe target area was assigned and centered over the platform coordinates. Each rat was subjected to a single 90-s videotaped probe trial swim. The total number of times each rat crossed the probe target area was recorded. During the probe trial swim, the swim speeds (m/s) were also calculated for each rat at each time point by determining the amount of time required for the rat to swim 0.5 m.

All data were analyzed for significance in a paired, two-way, repeated-measures Analysis of Variance (ANOVA), taking into consideration infection status (BDV-infected group vs. uninfected group) and set number. *Post-hoc* comparisons were assessed using the Student-Newman-Keuls method. In every case the acceptable level for statistical significance was $p < 0.05$.

Histologic Evaluation

After water maze testing, the rats were deeply anesthetized with Metofane inhalant anesthetic (Pitman-Moore, Mundelein, IL, USA) and killed. Brains and eyes were removed, immersion fixed in 4% paraformaldehyde and embedded in paraffin. The tissue was cut sagittally into 6-μm-thick sections and placed on glass slides.

To confirm infection status, whole paraffin-embedded sections of rat brain were examined for BDV protein expression by avidin-biotin immunohistochemistry (Vector, Burlingame, CA, USA) using mouse anti-BDV polyclonal antiserum as reported previously [14]. To confirm the presence or absence of DG neurons, avidin-biotin immunohistochemistry using rabbit anti-neuron-specific enolase (Chemicon, Temecula, CA, USA) and biotinylated mouse anti-rabbit polyclonal Ig (Vector) was performed. When appropriate, immunohistochemically stained sections were counterstained with hematoxylin.

Paraffin-embedded sections of rat eyes were examined for evidence of pathologic changes indicative of retinopathy, a condition reported in immunocompetent adult-infected rats but not in rats infected with BDV as neonates [31,43].

DG Neuron Density Estimates

Brain sections from infected, and uninfected rats were obtained at a standard distance from midline (2 mm) and were stained with hematoxylin and eosin. For each rat at each time point, DG neuronal density was estimated by counting nuclear profiles in three to five 40 × 40-μm squares of the DG. A nucleus was counted if it intersected the top or left side of each defined square. A Panasonic video camera and ColorSpace II/FX imaging board were used to overlay the microscopic image (100× objective) on a computer screen (Macintosh IIci). The 40 × 40-μm counting frame was drawn using NIH Image (1.44) or software for 3-D cell counting [63], kindly provided by P. Rakic (Section of Neurobiology, Yale School of Medicine). The estimates for healthy and pyknotic (dying, dead) neuronal profiles were recorded separately, and no corrections were made for double counting. Pyknotic

neurons were dead or dying cells characterized by the presence of nuclear chromatin condensation, convolution of nuclear membrane, irregular perikaryon and empty vacuolization. Data were analyzed for significance in a paired, two-way, repeated-measures ANOVA, taking into consideration infection status and age.

RESULTS

MWM Navigation Task

The performance in the MWM navigation task by the uninfected and BDV-infected rats is shown in Fig. 1. On postnatal day 43 (A), the mean escape latencies of the BDV-infected rats were significantly greater than those recorded for the uninfected rats in all four sets of trials; however, both groups of rats displayed comparable improvement in maze performance, calculated by comparison of the shortest mean escape latency of the four sets of five trials to the initial mean escape latency in the first set of trials. These results were demonstrated by *post-hoc* Student-Newman-Keuls all pairwise multiple comparison testing after a two-way repeated-measures ANOVA showed significant group, $F(1,39) = 13.18$, $p < 0.05$, and set, $F(3,39) = 8.08$, $p = 0.003$, effects but no significant Group × Set interactions, $F(3,39) = 0.95$, $p = 0.45$.

On postnatal day 53 (B), with exception of the first set, the mean escape latencies of the BDV-infected rats were significantly greater than those recorded for the uninfected rats. During the first set of trials, for unknown reasons, there was a high failure rate (7.5%) in the navigation task by the uninfected rats (i.e., the rats could not find the platform within 90 s). However, unlike the BDV-infected rats, latency in finding the platform by the uninfected rats significantly declined by the next set of trials and continued to decline in all subsequent sets of trials. These results were demonstrated by *post-hoc* Student-Newman-Keuls all pairwise multiple comparison testing after a two-way repeated-measures ANOVA that revealed significant group, $F(1,31) = 58.2$, $p = 0.005$, and Group × Set, $F(3,31) = 3.88$, $p = 0.049$, effects but no significant set effect, $F(1,31) = 2.85$, $p = 0.097$.

On postnatal day 72 (C), the mean escape latencies of the BDV-infected rats were significantly greater than those recorded for the uninfected rats in all four sets of trials. Again, in contrast to the BDV-infected rats, there was a significant decline in the latency of finding the platform by the uninfected rats from the first to last set of trials. These results were demonstrated by *post-hoc* Student-Newman-Keuls all pairwise multiple comparison testing after a two-way repeated-measures ANOVA that revealed a significant group effect, $F(1,31) = 106.9$, $p = 0.002$, but no significant set effect or Group × Set interaction (all $p > 0.05$).

It was apparent that the percent improvement in performance by the BDV-infected rats at each time point gradually worsened from postnatal day 43 (58 ± 11%) to 53 (37 ± 13%) to 72 (27 ± 12%; $p = 0.05$). In contrast, the percent improvement in the performance of the uninfected rats remained stable from postnatal day 43 (85 ± 3%) to 53 (88 ± 2%) to 72 (81 ± 4%; $p = 0.42$).

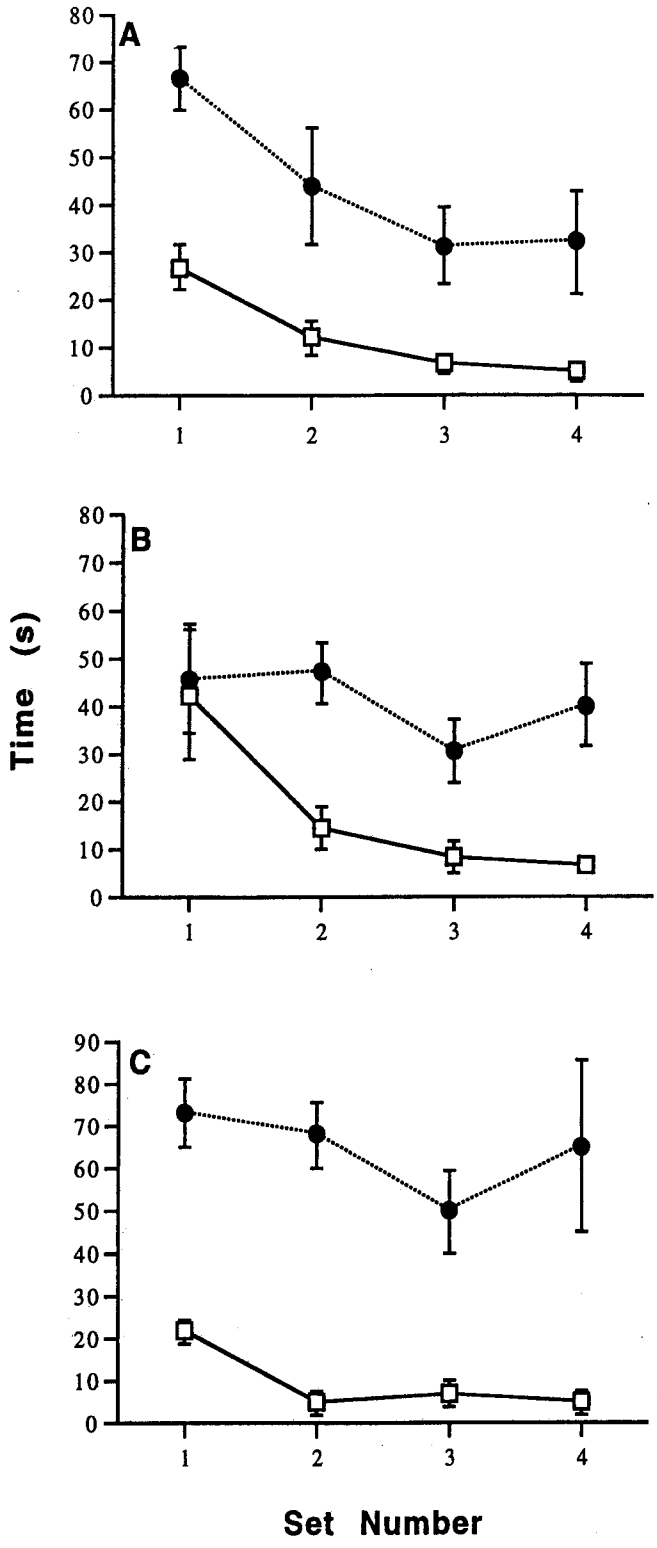

FIG. 1. Time (s) required for Borna disease virus-infected (solid circles) and uninfected (open boxes) rats to locate the submerged platform averaged over four successive sets of five trials at postnatal days 43 (A), 53 (B) and 72 (C).

MWM Probe Trial

Immediately after the four sets of five trials in the navigation task, each rat was subjected to a 90-s probe trial swim. In the probe trial, the escape platform (probe target) was removed, and spatial memory was indicated by the number of times each rat crossed the probe target area. Significantly fewer probe target area crossings were recorded for the BDV-infected rats compared with the uninfected rats, respectively, on postnatal days 43 (2.4 ± 0.8 vs. 9.0 ± 1.2; $p = 0.002$), 53 (2.3 ± 0.3 vs. 9.0 ± 0.8; $p < 0.001$) and 72 (0.75 ± 0.5 vs. 6.5 ± 0.9; $p = 0.001$) (Fig. 2). Additionally, probe trial performance by BDV-infected rats progressively worsened from postnatal day 43 to 72 ($p < 0.05$). No significant differences in the number of probe target area crossings were seen in the uninfected rat group over the three time points ($p = 0.16$).

During the probe trial, swim speeds were measured to assess the rats' ability to swim effectively. No significant differences in

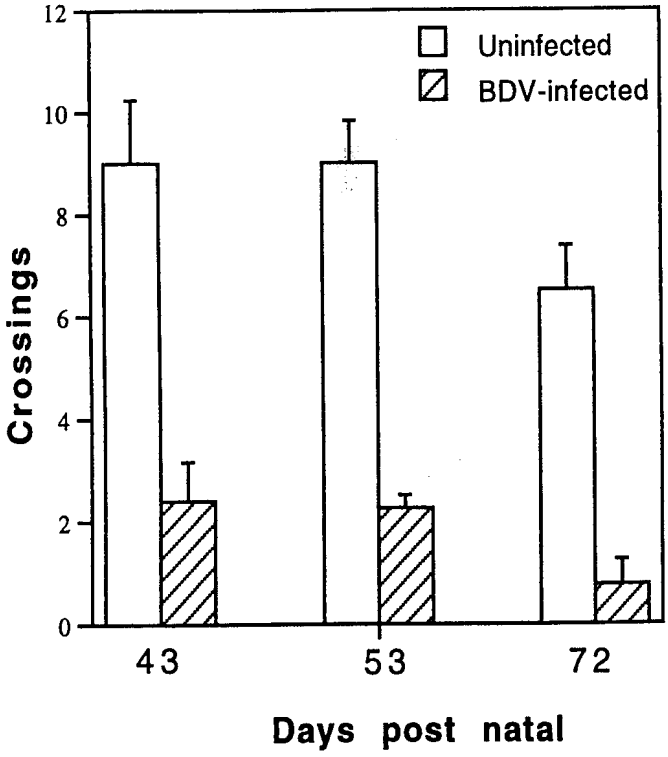

FIG. 2. Number of probe target area crossings by Borna disease virus-infected and uninfected rats at postnatal days 43 ($p = 0.002$), 53 ($p < 0.001$) and 72 ($p = 0.001$).

the average swim speeds were observed between BDV-infected and uninfected rats at 43 (0.38 ± 0.03 vs. 0.36 ± 0.02; $p = 0.65$) and 53 (0.46 ± 0.06 vs. 0.40 ± 0.03; $p = 0.38$) days of age. The average swim speed of the 72-day-old BDV-infected rats (0.29 ± 0.04) was significantly slower than that of the age-matched uninfected rats (0.43 ± 0.08; $p = 0.014$).

Histologic Evaluation/DG Neuron Density Estimates

Immunohistochemical staining of BDV-inoculated rat brain sections (data not shown) revealed widespread persistent BDV infection of the hippocampal formation including the entire DG as shown previously [15]. Despite extensive infection of the hippocampal formation, the most extensive damage appeared to be restricted to the DG neurons; subtle damage to other areas of the hippocampus including the portions of the pyramidal cell layer can also be seen. Hematoxylin and eosin-stained sections of BDV-infected rat brain confirmed the progressive deterioration of the DG over time postinoculation with significant degeneration noted on histologic evaluation by postnatal day 72 (Fig. 3). Dentate gyrus neuron density was estimated by counting the number of healthy and pyknotic (dead or dying) neuron nuclear profiles per mm^2 for each rat at each time point (Fig. 4). A significant increase in estimated pyknotic neuron density was seen in BDV-infected rats compared with uninfected rats at all time points tested (day 43, $p < 0.001$; day 53, $p = 0.015$; day 72, $p < 0.001$). Likewise, estimates of healthy DG neuron density progressively diminished in the BDV-infected rats from postnatal day 43 (1,592 ± 208/mm^2) to 72 (898 ± 134/mm^2; $p = 0.03$). No significant differences in estimates of healthy DG neuron density were detected in uninfected rat brain between any time points tested ($p > 0.05$). Immunohistochemical staining for neuron-specific enolase confirmed that the profiles estimated in these experiments were neurons (data not shown).

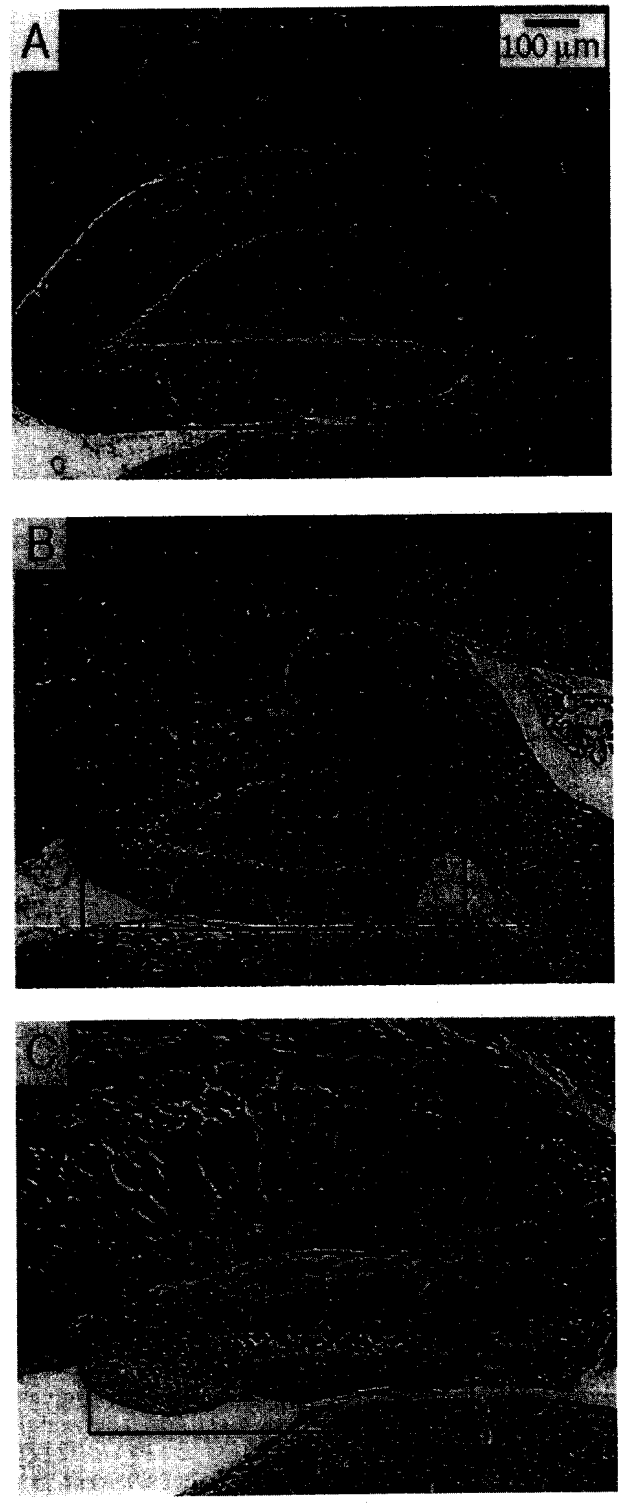

FIG. 3. Hematoxylin and eosin-stained sections of Borna disease virus (BDV) infected and control rat brain showing histopathology of the dentate gyrus (within box outline). (A) Uninfected brain at postnatal day 43; (B) BDV-infected brain at postnatal day 43; (C) BDV-infected brain at postnatal day 72. Note progressive deterioration of the dentate gyrus in BDV-infected brain from days 43 to 72 post inoculation.

None of the fixed paraffin-embedded eye sections of the BDV-infected rats used in this study showed any evidence of pathologic changes to the retina, supporting previous findings that vision in neonatally BDV-inoculated rats appears not to be compromised [43] (data not shown).

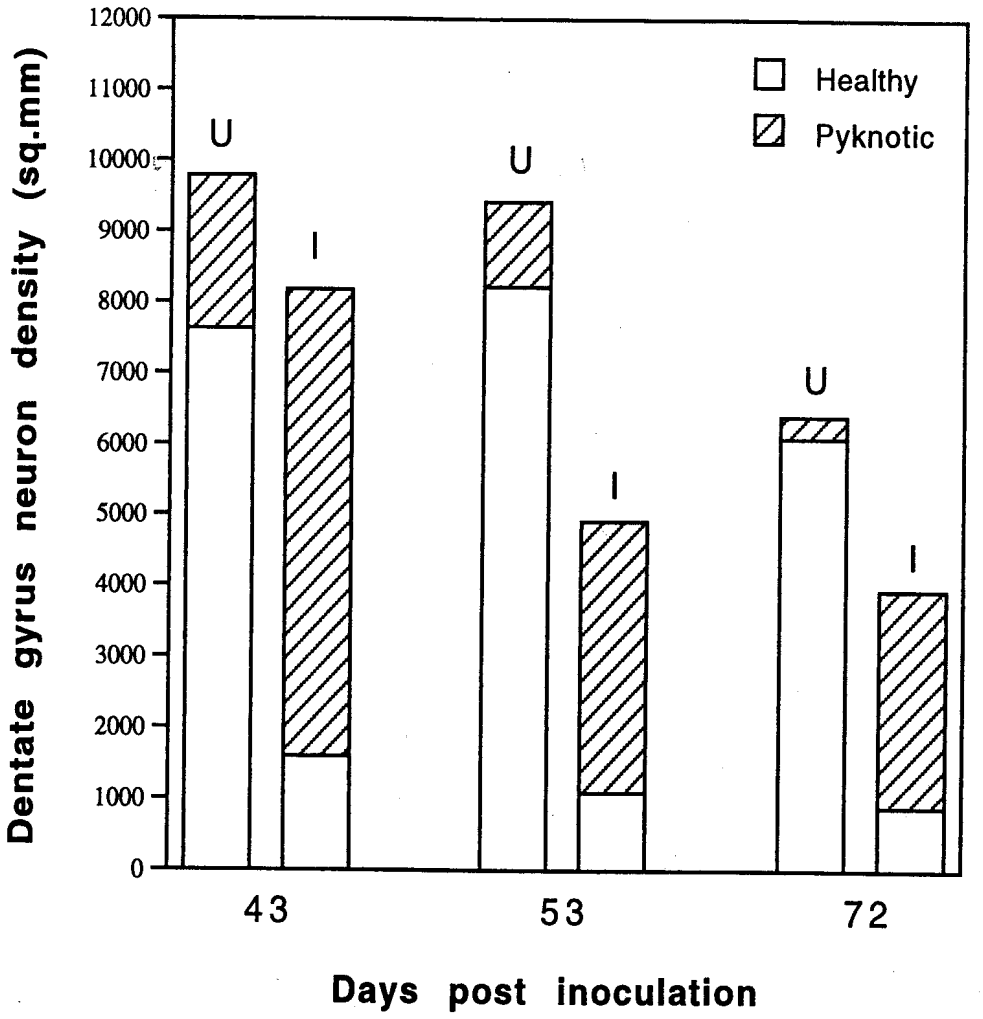

FIG. 4. Estimate of density of healthy and pyknotic dentate gyrus neuron cell profiles at postnatal days 43, 53 and 72. The number of pyknotic neurons increased significantly in Borna disease virus-infected (I) rat brain compared with uninfected (U) rat brain at each time point ($p < 0.001$, $p = 0.015$ and $p < 0.001$, respectively). Healthy dentate gyrus neurons progressively diminished in Borna disease virus-infected rat brain from postnatal day 43 to 72 ($p = 0.03$), whereas no significant difference was observed between these time points in uninfected rats brain ($p = 0.14$).

DISCUSSION

The role of the hippocampal formation (enterorhinal cortex, DG, hippocampus proper and subicular complex) in spatial learning and memory has been demonstrated by visual-cue maze testing of rats after selective or complete lesioning of this neuroanatomic region via surgical, chemical or hormonal means [1,10,16,33,40–42,44,50,57]. Of these structures, the DG has been shown to be particularly important in spatial learning and memory as demonstrated by MWM testing of rats after colchicine lesioning of DG granule cells [59]. Although viral infection of the hippocampal formation in rodents has also been associated with learning and memory deficits, for example, lymphocytic choriomeningitis virus [22,28], vesicular stomatitis virus [39], encephalomyocarditis virus [64] and BDV [23], virus-associated damage to the DG has not been reported concurrently with learning and memory deficits.

We have previously shown that BDV infection of neonatal rats leads to progressive degeneration of the DG in the absence of global inflammatory responses in seemingly clinically healthy animals [15]. These findings suggest the value of neonatally BDV-infected rats as a model in which to test spatial learning and memory after virally induced damage of the DG. In the work presented here, we tested neonatally BDV-infected rats in the MWM at various times postinfection (i.e., with increasing degrees of DG degeneration) to determine whether the gradual but dramatic degeneration of the DG was associated with progressive worsening of spatial learning and memory skills.

At each time point, the BDV-infected rats performed significantly worse than the uninfected rats, as manifested by increased escape latencies in the navigation task and decreased target platform crossings during the probe trial task. Additionally, at postnatal days 53 and 72, unlike the uninfected rats, the BDV-infected rats' performance in the navigation task failed to improve over the course of several sets of trials. Overall, MWM performance by the BDV-infected rats progressively worsened from postnatal days 43 to 53 to 72 in concert with the increasing degenerative effects of BDV infection of the DG neurons. Thus, BDV-induced damage to the DG may be linked to spatial learning and memory deficits in neonatally BDV-infected rats. Of note, other areas of the hippocampus appear to be affected by BDV, although this damage is subtle compared with the extensive neuronal lysis seen in the DG. Nonetheless, the possibility that subtle damage in other areas of the

hippocampus also contributed to the behavioral abnormalities cannot be ruled out.

In addition to the progressive degeneration of the DG, the cerebella of neonatally BDV-infected rats is also damaged [4,15]. Because the cerebellum has been shown to play a role in spatial learning and memory [19,36], we cannot also rule out that the damaged cerebella may have contributed to the deficits in spatial learning and memory skills. However, it is unlikely that the progressive worsening of performance over time was due to cerebellar injury because BDV-associated cerebellar damage is largely complete by the second to third postnatal week [4,15] and, unlike the DG, does not apparently progress over time [4]. It is plausible, however, that the slower average swim speed of the BDV-infected rats, compared with uninfected rats on postnatal day 72, may have led to increased escape latencies in BDV-infected rats. Although a possible factor in MWM performance at postnatal day 72, it is unlikely that swim speed *per se* was the sole determinant of poor performance in BDV-infected rats at all time points; at postnatal days 43 and 53, the swim speeds of BDV-infected and uninfected rats were similar, and yet, at both time points, the BDV-infected rats performed significantly worse than the uninfected rats.

Because the MWM paradigm is based on the rats' ability to use visual cues, it is possible that the progressive worsening in MWM performance over time by the BDV-infected rats could be due to retinal changes similar to the progressive immune-mediated retinopathy and blindness reported in immunocompetent adult-infected rats [31,43]. However, this is unlikely because neonatally BDV-infected rats do not generate severe cellular inflammatory responses to the virus and have been found not to suffer from blindness [43]. Indeed, upon histologic examination, the retinas of the BDV-infected rats used in this study appeared normal. It is important to note, however, that BDV may cause subtle damage to the retina not detectable by histologic examination and, thus, the possibility of such damage affecting the performance in the MWM is possible.

The correlation between DG neuron loss and memory deficits in aging humans [62] and the correlation between hippocampal neuron density and performance by rodents in the MWM have been reported [32,34]. However, it is important to point out that accurate neuron counting in this area is complicated by the presence of primordial stem cells in the adult rat CNS and by the continued neurogenesis and neuron migration to the rat DG up to 1 year after birth [48]. Nonetheless, the progressive and dramatic degeneration of the DG in neonatally BDV-infected rats is clearly evident in histologic sections and was confirmed by our neuron density estimates.

In light of the controversy regarding aging, memory and hippocampal neuron density [51], it is also important to note that viral infections and other insults to the brain can lead to biochemical and functional neuronal damage without resulting in cell death [9,45]. For example, in mice persistently infected with lymphocytic choriomeningitis virus, deficits in acquisition of spatial discrimination and reduced exploratory behavior correlated with virus infection of brain regions that are likely substrates for such deficits but did not result in neuronal loss or induction of an inflammatory response [22,28].

Whether the loss of persistently BDV-infected DG neurons was due to direct viral lysis of the infected cells or various indirect factors, such as immunologic clearance or release of cytokines, excitatory amino acids or stress hormones, remains unknown. Because neonatally BDV-infected rats are, in general, devoid of significant inflammatory responses to viral CNS infection, it is tempting to speculate that the striking DG degeneration may occur by loss of mature virus-infected neurons with simultaneous BDV-associated prevention of the normal replacement of DG neurons by postnatal neurogenesis and migration. This hypothesis is consistent with cerebellar injury in neonatally BDV-infected rats where there is evidence both of gradual death of BDV-infected mature neurons (Purkinje cells) and abnormal neurogenesis and/or migration of granule cell precursors leading to a substantial reduction in granule cells [5]. However, further studies will be needed to confirm this hypothesis.

Studies with the rat model of BDV infection provide new evidence to support the hypothesis that neurotropic viruses can cause specific behavioral abnormalities, even in the absence of encephalitis. Moreover, due to BDV's tropism for and specific damage of discrete neuroanatomic areas, BDV-induced behavioral disease manifestations are anatomically consistent with the observed neuroanatomic damage. Given the recent evidence for isolation of BDV from humans [8,21,54,60], understanding BDV disease pathogenesis may have direct application to human disease. Because psychiatric illnesses are usually not accompanied by encephalitis, viruses such as BDV that can cause subtle but clear behavioral disease without stimulating an intense and generalized inflammatory response are plausible etiologic agents for neurobehavioral disease in humans.

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