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Developmental alterations in serotoninergic neurotransmission in Borna disease virus (BDV)-infected rats: A multidisciplinary analysis

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Neonatal Borna disease virus (BDV) infection of the rat brain serves as a valuable model for studying the pathogenesis of neurodevelopmental abnormalities following early brain injury. Previous experiments have demonstrated significant alterations in regional tissue content of serotonin (5-HT) in neonatally BDV-infected Lewis rats. The present study sought to provide more insights into postnatal virus-associated alterations in 5-HT neurotransmission by evaluating the density of 5-HT$_{1a}$ receptors in the hippocampus and 5-HT$_{2a}$ receptors in the cortex, regional 5-HT tissue concentrations, behavioral responses to a 5-HT agonist, quipazine, and numbers of neurons in specific subfields of the hippocampus on days 7, 14, and 30 after neonatal BDV infection in Lewis rats. Neonatal BDV infection was found to be associated with a gradual increase in the density of 5-HT$_{2a}$ and 5-HT$_{1a}$ postsynaptic receptors followed by an elevation of 5-HT contents at both the levels of synaptic terminals (i.e., cortex and hippocampus) and cell bodies (i.e., raphe nuclei). In addition, there was an enhanced behavioral response to quipazine. Virus-associated neurochemical and behavioral changes were accompanied by a decline in the number of neurons in the dentate gyrus and in the CA1 field of the hippocampus. No change in the number of neurons in the CA3/2 field of the hippocampus was observed. The present pattern of BDV-associated alterations in 5-HT brain system along with available data from other laboratories suggest that BDV might compromise axonal transport and/or release of 5-HT, resulting in decreased 5-HT neurotransmission. *Journal of NeuroVirology* (2004) **10,** 267–277.

Keywords: animal model; Borna; developmental behavioral disorders; serotonin

Introduction

Several developmental behavioral disorders have been associated with early brain injury following exposure to environmental insults (Yolken and Torrey, 1995; Mohamed *et al*, 1993; Gillberg, 1999). Because studying the complex mechanisms of developmental abnormalities in humans is very difficult, animal models are often used to identify pathogenic events and associated neurobehavioral consequences and to search for novel therapeutic regimens (Bachevalier, 1994; Yeung-Courchesne and Courchesne, 1997; Lord *et al*, 2000).

Neonatal Borna disease virus (BDV) infection of rats is a valuable animal model of neurodevelopmental damage (Carbone *et al*, 1991; Hornig *et al*, 1999; Pletnikov *et al*, 2002a; de la Torre, 2002). In Lewis rats, neonatal intracranial inoculation with BDV, a 8.9-kb nonsegmented, negative-strand, enveloped RNA virus (Bries *et al*, 1994; Cubbit *et al*, 1994),

produces persistent infection of the brain without confounding global damage from encephalitis and meningitis (Narayan *et al*, 1983; Hirano *et al*, 1983). Neonatally BDV-infected rats exhibit distinct behavioral deficits that are similar to several symptoms observed in developmental behavioral disorders such as autism (Rapin and Katzman, 1998). Examples include locomotor hyperreactivity to novel/aversive stimuli (Dittrich *et al*, 1989; Bautista *et al*, 1994; Pletnikov *et al*, 1999a; Hornig *et al*, 1999), deficient learning and memory (Dittrich *et al*, 1989; Rubin *et al*, 1999), and abnormal social (e.g., play) interaction (Pletnikov *et al*, 1999b). Such behavioral alterations may be explained by BDV-induced selective developmental damage to the neocortex, hippocampus, and cerebellum (Carbone *et al*, 1991; Bautista *et al*, 1995; Hornig *et al*, 1999; Eisenman *et al*, 1999; Gonzalez-Dunia *et al*, 2000; Zocher *et al*, 2000), brain regions that have been implicated in pathology of developmental behavioral disorders (Kemper and Bauman, 1998; Courchesne, 2002; Kern, 2003). Additionally, regional alterations in chemical neurotransmission may contribute to behavioral deficits observed in neonatally BDV-infected rats (Pletnikov *et al*, 2000, 2002b).

Our previous studies have shown that compared to control animals, concentrations of serotonin (5-HT) were greater in cortex, hippocampus, and cerebellum of BDV-infected rats from postnatal day (PND) 30 and onwards (Pletnikov *et al*, 2000). In addition, the density of postsynaptic 5-HT receptors was increased in the cortex and hippocampus in BDV-infected rats on PND 120 (Pletnikov *et al*, 2002b). These neurochemical studies showed a seemingly paradoxical profile of 5-HT alterations, i.e., increased tissue content (i.e., presynaptic levels) and up-regulation of postsynaptic 5-HT receptors. It is possible that the elevated levels of 5-HT in the cortex and hippocampus of BDV-infected rats could reflect a relative increase in innervation of brain areas that became smaller due to virus-induced neuronal dropout. Similarly, the increased density of 5-HT postsynaptic receptors in BDV-infected rats may be due to a more compact distribution of receptors on a small number of surviving neurons in the cortex and hippocampus. Alternatively, neonatal BDV infection might affect axonal transport and/or release of 5-HT, leading to compensatory up-regulation of postsynaptic receptors and elevated synthesis of 5-HT. Notably, disturbances in axonal transport have been described for other neurotropic viral infections (e.g., Tomishima *et al*, 2001). As our previous neurochemical studies were performed at one time point, i.e., on PND 120, sorting out primary versus compensatory changes is difficult.

In the present study, effects of neonatal BDV infection on 5-HT tissue content and the density of 5-HT postsynaptic receptors were evaluated in the same animals on PNDs 7, 14, and 30 to elucidate a temporal relationship between these neurochemical parameters. In order to gain more insights into a structural basis of 5-HT alterations, we also performed quantitative evaluations of neuronal loss in different areas of the hippocampus, the region mostly affected by BDV (Carbone *et al*, 1991; Hornig *et al*, 1999; Weisenbock *et al*, 2000; Zocher *et al*, 2000). In addition, we assessed functional status of 5-HT neurotransmission by testing behavioral effects of a 5-HT agonist, quipazine.

The data presented here show that neonatal BDV infection induced an initial up-regulation of postsynaptic $5-HT_{2a}$ and $5-HT_{1a}$ receptors in cortex and hippocampus, respectively, followed by an increase in tissue content of 5-HT in the same brain areas. Virus-induced up-regulation of postsynaptic receptors was associated with a decrease in the number of neurons in the dentate gyrus and the CA1 field of the hippocampus. Compared to the control animals, the BDV-infected rats demonstrated enhanced responses to quipazine, suggesting increased sensitivity of 5-HT receptors.

Results

5-HT tissue concentrations and turnover
We measured tissue content of 5-HT and its metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA), in the cortex, hippocampus, striatum, and raphe nuclei in order to gain more information on presynaptic levels of the neurotransmitter in developing control and BDV-infected rats. Cortex and hippocampus were chosen for analysis because they represent two major brain regions primarily affected by the virus. Tissue content in raphe nuclei was measured to evaluate effects of BDV on 5-HT cell bodies, whereas striatum was assayed as a brain region relatively spared by neonatal BDV infection in Lewis rats (Hornig *et al*, 1999; Weissenbock *et al*, 2000).

5-HT tissue levels (Figure 1)*:* The concentrations of 5-HT increased with age in cortex, $F(2, 29) = 51.3$, $P < .001$; hippocampus, $F(2, 34) = 24.2$, $P < .001$; striatum, $F(2, 26) = 24.0$, $P < .001$, but not in raphe nuclei, $F(2, 29) = 1.3$, $P > .05$. BDV infection was associated with significantly increased 5-HT tissue concentrations in cortex, $F(1, 29) = 6.05$, $P = .022$; hippocampus, $F(1, 34) = 8.8$, $P = .006$; and raphe nuclei, $F(1, 29) = 12.5$, $P < .001$. BDV infection did not significantly affect 5-HT content in striatum, $F(1, 26) = 0.33$, $P > .05$. Planned *t* test showed a significant virus-induced elevation of 5-HT concentrations in hippocampus and raphe nuclei on PND 14, and in cortex, hippocampus, and raphe nuclei on PND 30, all *P* values $< .05$.

5-HIAA/5-HT ratio (Table 1)*:* 5-HIAA/5-HT ratio, as an index of 5-HT turnover (Zaszek *et al*, 1981), was measured in the brain regions on PNDs 7 to 30. There was a significant effect of the age on 5-HT turnover in cortex, $F(2, 25) = 3.54$, $P = .048$; hippocampus,
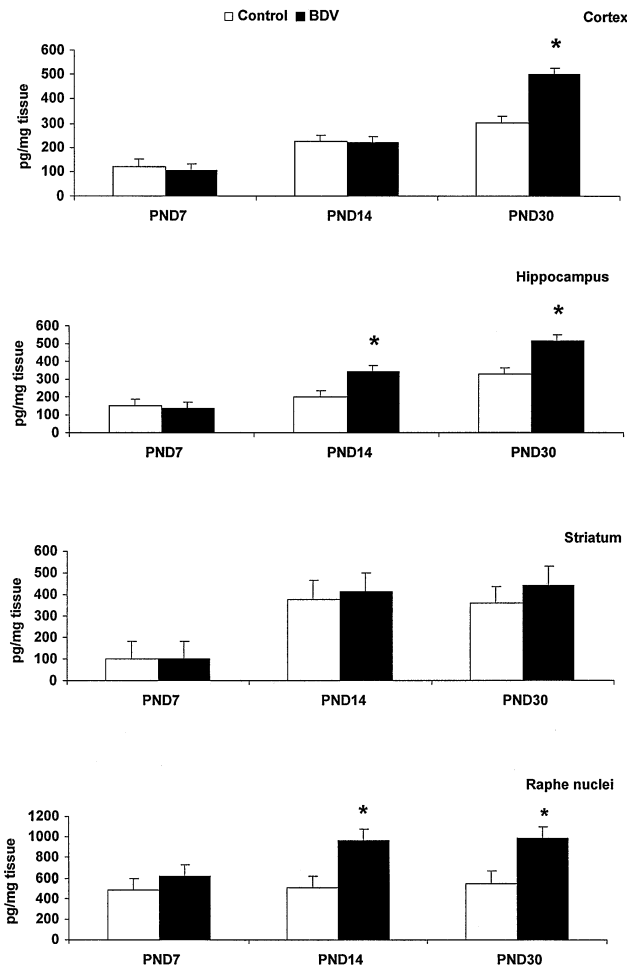
Figure 1 Tissue content of 5-HT in different brain regions in control (*open bar*) and BDV-infected (*solid bar*) rats on various postnatal days (PNDs). *$P < .05$ versus control values at the same time point and in the same brain region.

$F(2, 34) = 5.7$, $P = .008$; and raphe nuclei region, $F(2, 29) = 57.7$, $P < .001$; but no effect of age on 5-HIAA/5-HT ratio in the striatum, $F(2, 24) = 1.8$, $P > .05$. Neonatal BDV significantly affected 5-HT

Table 1 Effects of neonatal BDV infection on serotonin turnover (5-HIAAT/5-HT) in various brain regions

|	PND 7	PND 14	PND 30
Cortex			
Control	0.86 ± 0.1	0.83 ± 0.1	0.80 ± 0.1
BDV	0.91 ± 0.1	1.26 ± 0.1	0.80 ± 0.1
Hippocampus			
Control	0.77 ± 0.1	1.22 ± 0.1	0.95 ± 0.1
BDV	$1.09 \pm 0.1^*$	$1.53 \pm 0.1^*$	1.08 ± 0.1
Striatum			
Control	1.09 ± 0.3	1.73 ± 0.5	1.32 ± 0.5
BDV	1.34 ± 0.3	1.83 ± 0.5	1.87 ± 0.5
Raphe nuclei			
Control	0.57 ± 0.03	0.48 ± 0.03	0.98 ± 0.03
BDV	$0.53 \pm 0.03^*$	$0.32 \pm 0.03^*$	0.86 ± 0.03

PND = postnatal day; BDV = Borna disease virus.
*$P < .05$ versus control.

turnover in hippocampus, $F(1, 29) = 5.8$, $P = .022$ and raphe nuclei region, $F(1, 29) = 6.4$, $P = .018$. Planned *t* test revealed that compared to the control rats, 5-HT turnover was significantly higher in the hippocampus of the BDV-infected rats on PNDs 7 and 14, all *P* values $< .001$. In contrast, compared to the control rats, 5-HT turnover was significantly lower in the raphe nuclei of the BDV-infected animals on PND 7 and 14, all *P* values $< .001$. No effect of BDV infection on 5-HT turnover was observed in the cortex, $F(1, 25) = 3.0$, $P > .05$ or in the striatum, $F(1, 24) = 2.3$, $P > .05$, at any time point assayed.

Density of 5-HT$_{2a}$ receptors in cortex and 5-HT$_{1a}$ in hippocampus

We measured the density of postsynaptic 5-HT receptors in the cortex (5-HT$_{2a}$) and hippocampus (5-HT$_{1a}$) in control and BDV-infected rats at the same time points as those used to assess 5-HT concentrations. In this way, it was possible to evaluate whether alterations in the density of postsynaptic receptors precede the observed changes in levels of 5-HT in tissue.

Density of 5-HT$_{2a}$ receptors (Figure 2)*:* An analysis of the data for PND 7 revealed a significant effect of
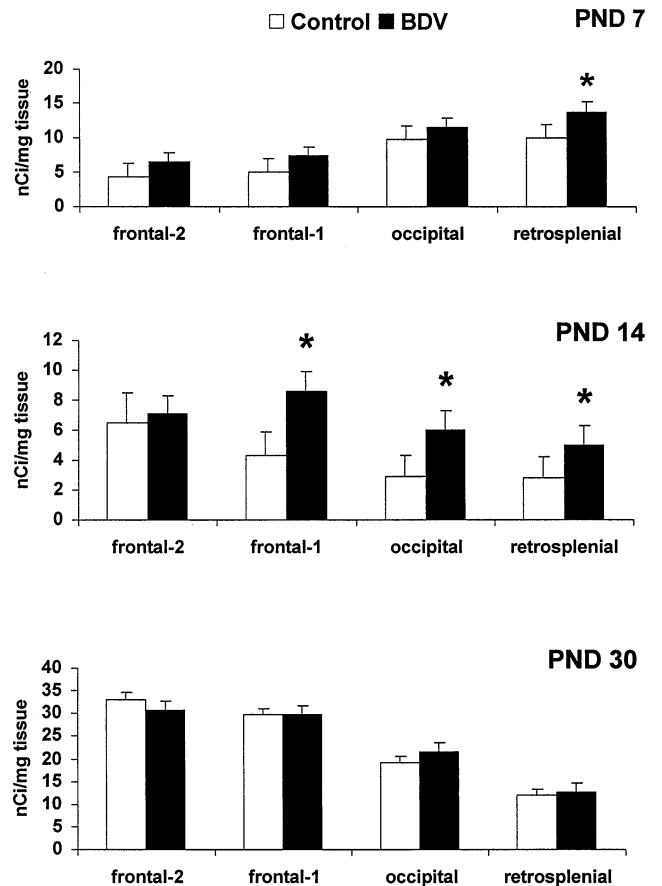
Figure 2 The density of 5-HT$_{2a}$ receptors in various cortical areas in control (*open bar*) and BDV-infected (*solid bar*) rats on various postnatal days (PNDs). *$P < .05$ versus control values at the same time point and in the same cortical area.

brain region, $F(3, 31) = 7.42$, $P = .001$. The highest density of the receptors was observed in the retrosplenial agranular cortex and the lowest density was found in frontal cortex, areas 2 and 1 (i.e., frontal-2 and frontal-1 on Figure 2, respectively). Neonatal BDV infection had a marginal overall effect on the density of 5-HT$_{2a}$ receptors in the cortex, $F(1, 31) = 4.2$, $P = .051$, with a trend towards increased density of 5-HT$_{2a}$ receptors in all brain regions of the BDV-infected rats compared to the control animals. Planned t test showed that the amount of bound ligand in the retrosplenial agranular cortex was significantly greater in the BDV-infected rats compared to the control animals, $P < .001$.

An analysis of the data for PND 14 also showed a significant effect of brain region, $F(3, 49) = 1.87$, $P < .05$, and a significant effect of infection status, $F(1, 49) = 5.76$, $P = .02$. Planned t test showed that compared to the control rats, the amount of the bound ligand was significantly greater in the BDV-infected rats in area 1 of frontal cortex, occipital cortex, and retrosplenial agranular cortex, all P values $< .05$.

An analysis of the data for PND 30 showed a significant effect of the brain region, $F(3, 46) = 61.72$, $P < .001$, and no effects of the infection status or the brain region by infection status interaction, $P > .05$.

Density of 5-HT$_{1a}$ receptors (Figures 3 and 4)*:* There were no virus-associated changes in the density of 5-HT$_{1a}$ receptors in any hippocampal areas assayed on PND 7, $P > .05$. The density of 5-HT$_{1a}$ receptors in the dentate gyrus of the hippocampus was greater than that in all CA subfields, $P < .05$.

On PND 14, there was a significant effect of hippocampal area, $F(3, 39) = 82.8$, $P < .001$, with the greatest binding in the dentate gyrus (DG) compared to all other areas, $P < .05$. Neonatal BDV infection produced a significant increase in the density of 5-HT$_{1a}$ receptors, $F(1, 39) = 34.5$, $P < .001$. Planned t test demonstrated that compared to the control animals, the density of 5-HT$_{1a}$ receptors were significantly greater in the BDV-infected rats in all areas of the hippocampus, all P values $< .05$ (Figure 4). In addition, a significant effect of region by infection status interaction, $F(3, 39) = 5.1$, $P = .005$, indicates that the binding was elevated to a greater extent in the BDV-infected rats in the DG and the CA3 area versus other areas of the hippocampus in the BDV-infected rats (Figure 4).

On PND 30, as with the other time points, the density of 5-HT$_{1a}$ receptors was greatest in the DG of the hippocampus compared to all other areas, $F(3, 27) = 49.5$, $P < .001$. There was also a significant effect of the infection status, $F(1, 27) = 18.8$, $P < .001$. Compared to the control animals, neonatal BDV infection was associated with a significantly greater density of 5-HT$_{1a}$ receptors in all but the CA-3 region of the hippocampus, P values $< .05$.
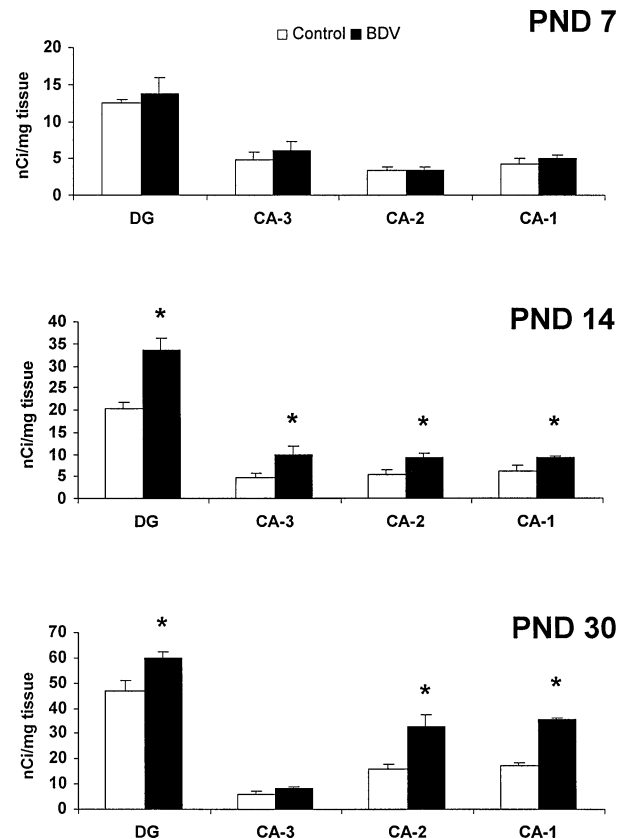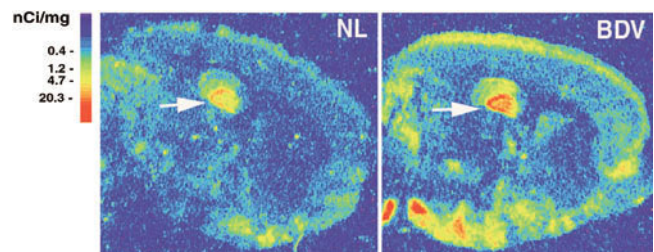

Figure 3 The density of 5-HT$_{1a}$ receptors in various hippocampal areas in control (*open bar*) and BDV-infected (*solid bar*) rats on various postnatal days (PNDs). *$P < .05$ versus control values at the same time point and in the same hippocampal area.

BDV-associated loss of neurons in the hippocampus (Figure 5)
In order to gain more insights into a relationship between numbers of surviving neurons and the density of postsynaptic receptors, we estimated BDV-associated neuronal loss in various areas of the hippocampus in control and BDV-infected rats on PND 30, the time point when the peak of cell death has been reported for the DG of the hippocampus (Weissenbock *et al*, 2000; Zocher *et al*, 2000).

Neonatal BDV infection produced a significant decrease in the volumes of the DG and the CA2/3 area,

Figure 4 Representative autoradiograms of specific binding of [³H]-8-OH-DPAT to 5-HT$_{1a}$ receptors in brains of control (NL) and BDV-infected (BDV) rats on PND 14. Note a robust BDV-associated increase in [³H]-8-OH-DPAT binding in the DG (*arrows*).
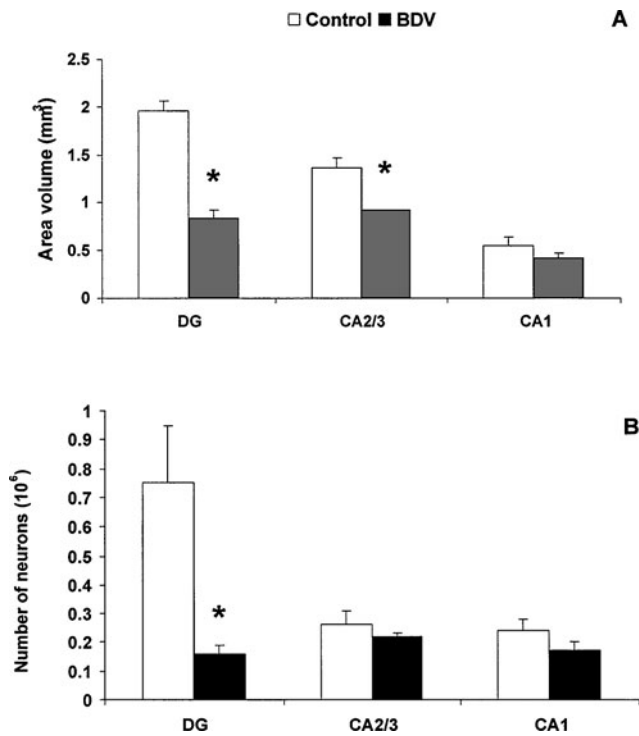
Figure 5 The volumes of the areas of the hippocampus (**A**) and the numbers of neurons (**B**) in the hippocampal areas in control (*open bar*) and BDV-infected (*solid bar*) rats on postnatal day 30. *$P < .05$ versus control values for the same brain area.

Figure 6 Effects of intraperitoneal injections of various doses of quipazine on horizontal (**A**), vertical (**B**), and stereotypic (**C**) locomotor activity in control (control) male and female and BDV-infected (BDV) male and female rats. Open bars = control rats; solid bars = BDV-infected. *$P < .05$ versus saline-treated rats of the same infectious and sex status.
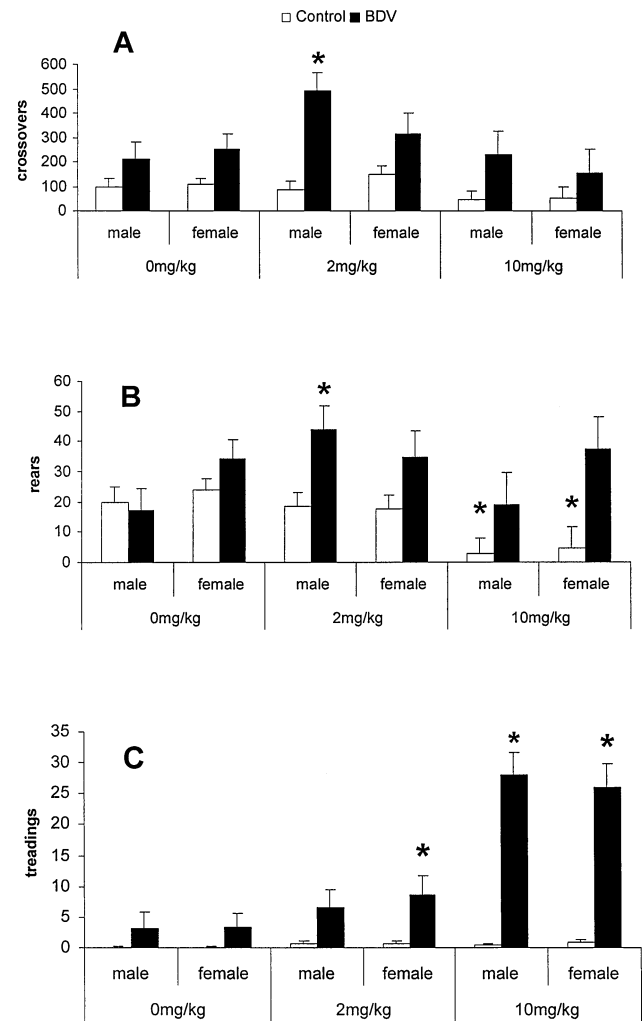
$P < .05$, whereas the volume of the CA1 subfield was not significantly altered by the infection on PND 30, $P > .05$ (Figure 5A).

Compared to control animals, there was a significant reduction in the number of granule neurons in DG on PND 30, $P < .001$. Although a trend toward fewer surviving neurons in the CA1 area of BDV-infected rats was observed, significant changes in the number of neurons in the CA1 and CA2/3 subfields of the BDV-infected animals were not found (Figure 5B).

Effects of a 5-HT agonist, quipazine (Figure 6)
We tested the behavioral effects of a nonselective 5-HT agonist, quipazine, in an open-field paradigm in order to evaluate the functional status of 5-HT receptors in control and BDV-infected rats.

Horizontal activity (Figure 6A): Intraperitoneal injections of quipazine produced a dose-dependent effect on horizontal locomotor activity in Lewis rats. An analysis of the data for horizontal activity showed a significant effect of infection status, $F(1, 55) = 39.7$, $P < .001$, and dose of the compound, $F(2, 55) = 5.1$, $P = .01$. Horizontal activity of the BDV-infected rats was greater than that of control animals in all treatment groups, all P values $< .05$. The compound significantly increased horizontal activity in the BDV-infected male rats at the dose of 2 mg/kg ($P < .05$). No effects were observed in male and fe-

male rats of both groups at the dose of 10 mg/kg, $P > .05$.

Vertical activity (Figure 6B): An overall analysis of the data for rearing showed a significant effect of infection status, $F(1, 55) = 19.7$, $P < .001$; dose, $F(2, 55) = 3.3$, $P = .047$; and an infection status by dose interaction, $F(2, 55) = 5.0$, $P = .011$. Quipazine injections increased rearing activity in the male BDV-infected rats at the dose of 2 mg/kg and decreased rearing activity in the control male and female animals at the dose of 10 mg/kg, $P < .05$.

Treading (Figure 6C): Injections of quipazine produced a strong behavioral response, i.e., front paw treading that is characteristic of "serotonin" behavior (Green and Backus, 1990). An overall analysis of the data for treading demonstrated significant effects

of infection status, $F(1, 55) = 83.3$, $P < .001$; dose, $F(2, 55) = 29.2$, $P < .001$; and an infection status by dose interaction, $F(2, 55) = 27,66$, $P < .001$. For the control rats, both doses of the compound induced a slight but not significant increase in numbers of treading, $P > .05$. In contrast, quipazine significantly increased numbers of treading in the BDV-infected animals. Planned t tests showed that 2 mg/kg of quipazine produced significantly more treading in the BDV-infected female but not male rats compared to the respective saline-treated BDV-infected rats, $P < .05$. In addition, a particularly strong response was observed in the BDV-infected rats after injection of 10 mg/kg of the compound, $P < .05$.

Discussion

The present data demonstrate that neonatal BDV infection of the brain produces a number of developmental alterations in 5-HT neurotransmission. Specifically, there was a gradual BDV-induced increase in the density of postsynaptic receptors that was followed by an elevation of tissue content of 5-HT at levels of synaptic terminals and cell bodies and was associated with enhanced behavioral responses to a 5-HT agonist, quipazine. The observed neurochemical and behavioral changes were accompanied by a decline in the number of neurons in the dentate gyrus, a trend towards a decline in the number of neurons in the CA1 field of the hippocampus, and the lack of changes in the number of neurons in the CA3/2 field of the hippocampus. Neonatal BDV infection also produces a decrease in the volume of the dentate gyrus and the CA3/2 field of the hippocampus but not in the CA1 field of the hippocampus.

Several putative mechanisms could be proposed to explain the observed profile of neuropathological, neurochemical, and behavioral alterations in developing Lewis rats neonatally infected with BDV. It is possible that BDV leads to a dropout of postsynaptic neurons (e.g., in the dentate gyrus of the hippocampus and cortical neurons), without affecting presynaptic terminals of ascending monoaminergic projections to the forebrain regions. In this case, elevated concentrations of 5-HT would reflect a relative increase in innervation of smaller cortical and hippocampal brain areas. A similar explanation has been proposed for the effects of methylazoxymethanol acetate (MAM), a compound that kills proliferating neurons without affecting presynaptic endings (Sanberg *et al*, 1987; Kodama *et al*, 2000). However, this hypothesis cannot explain the increased density of postsynaptic receptors, elevated content of 5-HT at the levels of cell bodies (i.e., raphe nuclei), and enhanced responses to quipazine. In fact, if a MAM-like phenomenon took place, we would expect to see compensatory down-regulation of postsynaptic receptors and decreased levels of 5-HT in the

nuclei raphe region as it has been found in MAM-treated rats (Beaulieu and Coyle, 1982; Kodama *et al*, 2000).

Alternatively, one could hypothesize that BDV infection of neurons and subsequent spreading of the virus along neural pathways may affect neurotransmission. In the host, BDV has been proposed to spread trans-synaptically along nerve fibers, suggesting the possibility of "highjacking" synaptic apparatus and/or axonal transport machinery (Carbone *et al*, 1987; Tomishima *et al*, 2001). In a similar vein, trans-synaptic spreading of BDV might lead to compromised synaptic vesicular mechanisms, possibly resulting in decreased neurotransmitter release. This pathogenic process would explain elevated concentrations of 5-HT at both presynaptic terminals and cell bodies. *In vivo* microdialysis experiments may be helpful to test a hypothesis of attenuated release of 5-HT. There have been several reports describing possible axonal and synaptic damage in BDV-infected rats. For example, Gies *et al* have found degenerating BDV-immunopositive axons with damaged organelles, filaments, or tubules and accumulations of smooth membranes in the subcortical areas 15 days after infection with BDV in adult-infected rats (Gies *et al*, 1988, 2001). Another study has found that the corpus callosum and cortex of 45-day-old neonatally BDV-infected rats exhibited patchy immunoreactivity for synaptophysin (SYN) with immunostaining accumulated in axonal "blobs," suggesting abnormal axoplasmic flow and/or impaired transport of SYN to synaptic terminals (Gonzalez-Dunia *et al*, 2000). Additionally, a most recent *in vitro* study has demonstrated significant alterations in the ultrastructure of the synapses, including clustering synaptic vesicles in the bouton of infected neurons in primary neuronal culture of the hippocampus (Hans *et al*, 2004). The hypothesis of virus-induced damage to axonal transport and the synaptic apparatus is consistent with virus-associated up-regulation of postsynaptic 5-HT receptors and their sensititzation to the effects of quipazine as well as with BDV-elevated concentrations of 5-HT at the cell bodies, (i.e., raphe nuclei). All these changes may represent compensatory changes aimed at restoring 5-HT neurotransmission. Future experiments utilizing cell culture models are needed to further characterize the mechanisms of direct effects of BDV on the axonal and synaptic processes.

Although BDV rapidly spreads throughout the brain within a few days after inoculation with comparable viral titers in the cortex, hippocampus, hypothalamus, and striatum (Morales, 1988; Bautista *et al*, 1995; Gonzalez-Dunia *et al*, 2000; Weissenböck *et al*, 2000), it is conceivable that variability in site-specific virus concentrations exists at very early time points post inoculation, and this may have contributed to the observed brain region–specific neurochemical and neurological alterations (i.e., greater damage to the hippocampus versus striatum).

Alternatively, these differences could be related to on-going processes of neuronal maturation in those brain regions, leading to their enhanced vulnerability to direct effects of viral infection.

In addition to direct viral effects of BDV on synaptic neurotransmission, indirect mechanisms of BDV-induced neural dysfunction are worth considering. In this context, HIV-induced effects of soluble neurotoxic factors may provide one possible model (Rausch and Davis, 2001). Given that BDV infection of the brain produces robust and widespread response of the resident immune system of the brain, i.e., astrocytosis and microgliosis (Carbone *et al*, 1989; Sauder and de la Torre, 1999; Hornig *et al*, 1999), it cannot be ruled out that secreted soluble proinflammatory molecules and ensuing oxidative stress in neurons and their processes might affect synaptic transmission (Medana and Esiri, 2003). For example, viral infection–associated death of glia or loss of glial function, (e.g., inhibited glutamate up-take) could affect axons (Liu *et al*, 1999). Additionally, secreted neurotoxic factors produced in response to infectious agents and/or damage the neural tissue can devastate intra-axonal cytoskeletal and organelle function (as reviewed by Medana and Esiri, 2003). Thus, although it is unlikely that all neurological and behavioral manifestations of neonatal BDV infection are due to putative axonal injury *per se*, it is conceivable that several major neurochemical alterations result from axonal injury in BDV-infected animals.

Indirect mechanisms of neuronal dysfunction in BDV-infected rats could also be responsible for the observed regional differences in neuronal and neurochemical alterations. For example, compared to the extent of functional damage in the striatum, greater neurological and neurochemical alteraitons in the cortex and hippocampus might result from more extensive gliosis in these areas (Sauder and de la Torre, 1999; Hornig *et al*, 1999). In addition, extensive BDV-associated abnormalities in the cortex and hippocampus could be related to enhanced vulnerability of these postnatally maturing brain areas to detrimental effects of proinflammatory factors (Dammann and Leviton 1997).

BDV-associated release of cytokines could also affect thermoregulation in BDV-infected rats either directly (Luheshi, 1998) or indirectly via alterations in serotonin neurotransmission (Gemma *et al*, 1997; Oka *et al*, 2001). Changes in body temperature may potentially influence behavioral abnormalities in infected rats. Although there have been no published reports on effects of neonatal BDV infection on body temperature in rats and body temperature was not assessed here, one cannot rule out the possibility that BDV infection may be associated with hyperthermia, which may contribute to some of the observed behavioral abnormalities. Similarly, because quipazine administrations have been shown to produce hyperthermia (Czyrak *et al*, 1991), one could speculate that

quipazine-induced decrease in locomotor activity at the dose of 10 mg/kg was, at least in part, due to increased body temperature.

Taken together, the present findings allow us to propose a hypothetical chain of alterations in 5-HT neurotransmission. Early stages of BDV infection may be associated with impairment in axonal transport and diminished release of neurotransmitter due to utilization of the axonal machinery by the virus (PNDs 7 to 14). These early events are characterized by up-regulation of postsynaptic receptors and the beginning of elevation of presynaptic concentrations of 5-HT (PNDs 14 to 30) in order to compensate for decreased neurotransmission. Simultaneously, virus-associated neuronal elimination occurs in some brain regions (e.g., dentate gyrus of the hippocampus). BDV-induced neuronal loss is accompanied by a decrease in the volume of an affected brain region. Consequently, the virus-related density of postsynaptic receptors becomes comparable to control levels because fewer surviving neurons remain, whereas presynaptic contents of 5-HT continues to increase to compensate for attenuated neurotransmission (PND 30 and onwards).

The present work also highlights the importance of modeling the pathogenic mechanisms in animals in order to appreciate complex evolving pathological changes that are not readily accessible to analysis using postmortem samples or single–time point evaluation in humans (Cook and Leventhal, 1996; Chugani, 2002). In this context, animal models can help identify primary and secondary alterations to indicate the mechanisms underlying variable outcomes. For example, the findings of increased blood concentrations of a neurotransmitter or its metabolites are usually interpreted as an indication of increased neurotransmission, whereas our results show that increased concentrations measured at one time point or without corroborating evaluations of several indexes of a system may not be indicative of elevated neurotransmission but rather could reflect compensatory changes.

In conclusion, the present data showed that neonatal BDV infection leads to significant alterations in 5-HT neurotransmission. Specifically, our findings appear to indicate that the viral infection produces decreased 5-HT neurotransmission, possibly resulting from affected synaptic release and/or axonal transport.

Materials and Methods

Animals
Pregnant Lewis rats (16 to 18 days of gestation) were purchased for the present study (Harlan, Indianapolis, IN). All rat pups were born in the animal facility at CBER/FDA, Bethesda, MD.

Mothers and their litters were housed in 45 × 26 × 23-cm pan-type polypropylene cages with paper-chip

bedding and an overhead wire grid supporting food pellets and a water bottle. Cages containing infected animals were kept in a DUO-FLOU biosafety cabinet (Bio-Clean Lab Product Inc., NJ). The sham-inoculated rats were kept in the same room. Rats of all the groups were maintained on a 12/12-h light/dark cycle (lights on at 8 AM). Pregnant rats of all the groups had free access to food and water. After weaning, on PND 23, rats continued to have free access to food and water. Room temperature was maintained at approximately 21°C.

Pups were inoculated intracranially under hypothermia anesthesia with 26-gauge needles within 24 h of birth either with 0.02 ml of infectious inoculum containing 50% tissue culture infective doses (TCID/50)/mL of He-80 BDV strain or 0.02 ml of uninfected inoculum as described previously (Carbone *et al*, 1991). For intracranial inoculation, a pup was taken out of the home cage and placed on ice. After an injection, a rat pup was warmed with a warm cloth, and returned to the home cage. BDV stock was prepared from homogenized BDV-infected rat brain tissue as described earlier (Carbone *et al*, 1991).

In order to minimize any injection site reaction from potentially affecting measured virus-associated neurochemical and morphological alterations, inoculation of the virus was performed in an alternating manner so that one half of the rat pups were inoculated in the right hemisphere, and the other half were inoculated in the left hemisphere. Hemisphere selection for different experiments was made randomly so that approximately equal numbers of inoculated and uninoculated hemispheres were included in any assay.

For all the experiments no more than two pups from a single litter were used per each test group. On PNDs 7 and 14, a subset of the rat pups were euthanized for measuring 5-HT tissue content and the density of 5-HT postsynaptic receptors. On PND 30, the rats were tested in the behavioral experiments and then euthanized for neurochemical and neuroanatomical studies as described below.

Neurochemical experiments
A temporal correlation between tissue concentrations of 5-HT and the density of postsynaptic 5-HT receptors was evaluated on PNDs 7, 14, and 30 in BDV-infected and sham-inoculated control animals. For each time point, three male and two female ($n = 5$) rats were used per treatment group. The PND-30 group consisted only of rats given saline injections as described in the behavioral experiments.

Rats were euthanized by decapitation, brains were removed and cut sagittally in halves. One hemisphere was rapidly dissected on ice for 5-HT tissue content analysis, and the other hemisphere was frozen on dry ice and used for receptor autoradiography analysis. In this way, tissue content and density of the receptors were measured in the same animals.

Tissue content measurement: The following brain regions were dissected for analyses: frontal cortex, hippocampus, striatum, and raphe nuclei region. Samples were stored at −70°C until assay. Regional concentrations of 5-HT and its metabolite 5-HIAA were measured in the dissected brain regions by high-performance liquid chromatography with electrochemical detection (HPLC-ED) (Zaczek and Coyle, 1981). Monoamine peaks in chromatograms of samples were identified by their retention times. Monoamine concentrations were expressed as pg/mg tissue.

Quantitative receptor autoradiography: Frozen brains were sectioned sagittaly at 16 μm. Sections were thaw-mounted onto gelatin-coated slides and stored at −80°C until assay.

For autoradiographic determination of 5-HT$_{1a}$ receptor binding, sections were thawed at room temperature and preincubated for 30 min in 50 mM Tris buffer (pH 7.4 at 25°C). Sections from each group were then incubated for 60 min with the saturating concentration (2 nM) of [^{3}H]-8-OH-DPAT = 8-hydroxy-2-di-n-propylaminotetral (NEN, Boston, MA) in the presence or absence of unlabeled 1 μM 5-HT (Sigma, St. Louis, MO) to estimate nonspecific binding. The assay buffer consisted of 50 mM Tris with 0.1% ascorbic acid (*w/v*), 4 mM CaCl$_2$, and 10 μM pargyline (Sigma) (Knapp *et al*, 1998). Slides were then quickly dipped once in ice-cold buffer to rinse, washed twice (5 min each) in ice-cold assay buffer, rinsed for 15 s in ice-cold distilled water to remove buffer salts, and dried with cool air. Slides of total binding ([^{3}H]-8-OH-DPAT only) and nonspecific binding ([^{3}H]-8-OH-DPAT with 5-HT) from each group of rats were then apposed to radiosensitive ^{3}H-Hyperfilm film along with ^{3}H microscales (Amersham, Arlington Heights, IL) for 14 days. The density of 5-HT$_{1a}$ receptors was assessed on slides corresponding to plate 82 on the rat brain atlas (Paxinos and Watson, 1986).

For autoradiographic determination of 5-HT$_{2a}$ receptor binding, sections were thawed and preincubated for 15 min in 50 mM Tris buffer (pH 7.4 at 25°C). Sections were then incubated for 120 min with 2 nM [^{3}H]-ketanserin (NEN, Boston, MA) and 100 nM prazosin (Sigma) in the presence or absence of 10 μM methylsergide (Sigma) to determine nonspecific binding (Knapp *et al*, 1998). Slides were then rinsed in the Tris buffer for 1 min prior to two 10-min washes in the Tris buffer followed by a brief rinse in distilled water to remove buffer salts. Final processing of slides proceeded as described for 5-HT$_{1a}$ receptor autoradiography, except that films were exposed for 21 days. The density of 5-HT$_{2a}$ receptors was assessed on slides corresponding to plate 82 on the rat brain atlas (Paxinos and Watson, 1986).

Both [^{3}H]-8-OH-DPAT and [^{3}H]-ketanserin binding was measured using a software, "the Scion Image

Beta 4.02 for Windows" (Scion Corporation, 2000). Standard curves were generated from ^{3}H microscales (Amersham). The binding data are expressed as nCi/mg tissue (mean ± SEM).

Effects of quipazine
In order to evaluate putative BDV-associated alterations in the functional status of 5-HT receptors, we studied effects of a 5-HT agonist, quipazine (Spear and Ristine, 1981; Enters and Spear, 1988). The compound was dissolved in 50 μl of dimethyl sulfoxide followed by diluting in saline to make the injected volume 0.5 ml. The compound was injected intarperitoneally at doses of 2 and 10 mg/kg. The following groups of rats were tested. Control rats included saline-treated, $n = 12$ (six males and six females), 2 mg/kg-quipazine-treated, $n = 9$ (five males and four females), and 10 mg/kg-quipazine-treated, $n = 6$ (three males and three females) rats. BDV-infected rats included saline-treated, $n = 14$ (seven males and seven females), 2 mg/kg-quipazine-treated, $n = 9$ (four males and five females), and 10 mg/kg-quipazine-treated, $n = 6$ (three males and three females) rats. Rats were tested in an open field 10 min after injections.

The open-field arena consisted of a Plexiglas square box (60 × 60 × 50 cm) with transparent walls. The floor was divided into 36 sections of equal area by a series of solid lines forming squares. A 100-watt white spotlight brightly illuminated the apparatus. Rats were not habituated to the experimental box. After a rat was placed in the open field arena, behaviors were videotaped for 10 min and scored later for horizontal locomotor activity (number of squares crossed), rearing activity, and stereotypic "serotonin" responses, including forepaw treading, hind-limb abduction, flat body posture, head weaving or twitching, and wet dog shakes (Ristine and Spear, 1985; Green and Backus, 1990).

Stereology-based count of neurons
in the hippocampus
Saline-treated uninfected ($n = 3$) and BDV-infected ($n = 3$) male Lewis rats were randomly preselected from the pool of rats used in behavioral experiments described in this study. Rats were deeply anesthetized with ether (Pitman-Moore, Mundelein, IL) followed by Euthasol, and perfused with phosphate buffered saline (pH = 7.4) followed by 4% paraformaldehyde. The cerebrum was dissected, paraffin-embedded, and cut sagittally into 25-μm-thick sections. The numbers of neurons in the hippocampal DG and CA2/3 and CA1 areas were estimated in the right hemispheres in uninfected and BDV-infected rats. The fractionator sampling scheme and optical dissector were used to quantitatively measure neuronal loss. These measurements were performed using an Olympus microscope with a computer-driven X,Y,Z-stage con-

troller (ASI, Eugene, OR) and stereology software (Stereologer; SPA, Alexandria, VA). Fifteen sagittal sections through the hippocampus were selected using a systematic random sampling scheme (e.g., every 15th section with a random start beginning from the most lateral tissue section). Sections were deparafinized and stained with cresyl violet and viewed at a magnification of 46×. Estimates of the numbers of neurons were made by counting neurons within a defined volume in each region using an optical dissector. The dimensions of the optical dissector for DG granule cells were height 10.00 μm, guard height 5 μm, spacing 200 μm for NL, and 100 μm for BDV-infected brains. The spacing of the optical dissectors was decreased for BDV-infected brains in order to increase the sampling frequency to keep the coefficient of error (CE) below 10%. The dimensions of the optical dissector for the CA1 and CA2/3 areas in uninfected and BDV-infected rats were as follows. CA1: frame area 738 μm^2, height 12 μm, guard height 2 μm, spacing 200 μm; CA2/3- frame area 553 μm^2, height 12 μm, guard height 2 μm, spacing 250 μm. The volume and spacing of the optical dissectors was adjusted to obtain a CE below 10% for the estimates of pyramidal neuron number. The boundaries of the DG and CA1 and CA2/3 areas were defined as described by West *et al* (1991).

To count neurons, an image of counting regions was displayed on the video monitor and a square counting box superimposed on the image using the stereology software "Stereologer" (SPA). Sections were viewed with a 100× objective (final magnification on the computer screen 2200×). The depth of the box was measured with the z-axis encoder attached to the focusing knob. Nomarski optics was used to optically section the tissue within the box. The nuclei of neurons were darkly stained with cresyl violet and were used as the criterion for determining if a cell should be counted. A cell was counted if the top of its nuclei came into focus within the box or touched the top and the right edges of the square frame or the lower surface of the box. Nuclei that touched the bottom and the left edges of the box and the upper surface of the box were excluded.

Volume measurements
The brain hemispheres used for counting neurons were also utilized for measuring volumes of DG and CA2/3 and CA1 areas with Cavalieri point counting using the stereology software "Stereologer" (SPA) as described in West *et al* (1991). The average coeffecient of variance for volume estimates in rats was .05.

Anti-BDV immunohistochemistry
Adjacent unused sections from the counting experiments were deparafinized and stained by avidin-biotin immunohistochemistry (Vector, Burlingame, using polyclonal horse anti-BDV antibodies followed by biotinylated anti-horse immunoglobulin

CA) (IgG) (Vector) as described previously (Carbone *et al*, 1991).

Statistical analyses

The data are presented as mean ± SEM. The data for tissue content were analyzed by two-way analysis (ANOVA) with infection status and age as independent variables separately for each brain region assayed. The data for receptor radioautography were analyzed by two-way ANOVA with infection status and brain region as independent variables separately for each time point. The data for effects of quipazine were analyzed by two-way ANOVA with infection status and dose of the compound as independent variables. Additionally, effects of sex on quipazine actions were analyzed separately in the BDV-infected and uninfected control rats using two-way ANOVA with sex and dose of the compound as independent variables. One-way ANOVA with infection status as an independent variable was used to analyze effects of neonatal BDV infection on the number of neurons in each hippocampal area separately. Tukey's tests or planned *t* tests tests were used when applicable. If the data did not pass the normality test and/or equal variance test, the data were subjected to the rank transformation test, and ANOVAs were rerun on the transformed data. A *P* < .05 was considered as the criterion for statistical significance.

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