[Frontiers in Bioscience 7, d593-607, March 1, 2002]

BORNA DISEASE VIRUS INFECTION OF THE NEONATAL RAT: DEVELOPMENTAL BRAIN INJURY MODEL OF AUTISM SPECTRUM DISORDERS

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1. ABSTRACT

Autism spectrum disorders (ASD) have been the focus of a great deal of research and clinical speculation. This intense interest relates to both the perplexing pathogenesis and devastating consequences of these disorders. One of the obstacles to understanding the pathogenesis of autism and its efficient treatment has been the paucity of animal models that could be used for hypotheses-driven mechanistic studies of abnormal brain and behavior development and for the pre-clinical testing novel pharmacological treatments. The present review provides a detailed analysis of a new animal model of ASD. This model utilizes neonatal Borna disease virus (BDV) infection of the rat brain as a unique experimental teratogen to study the pathogenesis of neurodevelopmental damage. For more than a decade, studies of the BDV animal model have yielded much insight into the pathogenic processes of abnormal brain development and resulting autistic-like behavioral abnormalities in rats. The most recent experiments demonstrate the utility of the BDV model for studying the pathophysiological mechanisms of the gene-environment interaction that determines differential disease outcomes and variability in responses to treatments.

2. INTRODUCTION

Investigating the pathogenesis of behavioral disease following virus-induced developmental brain damage is a daunting task. When studying the mechanisms

of behavioral and neurological developmental injury, one must take into account the neurodevelopmental stage of the brain at the time of infection (1-2), the effects of the virus replication and immune response on the development of brain regions undergoing neurogenesis and migration (1-3), the long-term consequences of continuing damage by a persistent viral infection on the function of the nervous system during adult life and aging (2), and the recovery of damaged brain regions over time (1,4).

In autism spectrum disorders (ASD) and other diseases of neurological development, the paucity of economical, relevant small animal models significantly inhibits the experimental study of the pathogenesis of these childhood behavioral disorders (5-7). Developing animal models for psychiatric disturbances is an extremely difficult enterprise (8-9). There have been suggestions that an ideal animal model should resemble the disease it models in its symptomology, etiology, biochemistry, and treatment (10). Unfortunately, with autism as with many other childhood behavioral disorders, relatively little is known about the etiology and the pathogenic mechanisms (5,11,13). Moreover, several key symptoms of ASD are usually of a cognitive and language nature, making it almost impossible to model them in animals (5,6,12).

Searching for ideal models bears the great risk of becoming trapped in a vicious circle. On the one hand, we need information about the disease in order to develop a good animal model. On the other hand, we want to develop animal models in order to increase our knowledge about the disease we are modeling. Assuming that valuable animal models do not have to exactly mimic every feature of complex human syndromes is a more productive approach. In this case, a good animal model will effectively reproduce several essential pathogenic processes and symptoms of the human disease. Modeling key pathophysiological mechanisms would allow us to conduct hypothesis-driven mechanistic studies that could provide better insight into the complex picture of the pathogenesis of a disease.

3. ANIMAL MODELS OF PSYCHIATRIC DISORDERS

There are different approaches to modeling neuropsychiatric diseases in animals. The first class of animal models has predictive validity. The validity of these models is only based on their ability to predict the effects of pharmacological drugs. These models are sometimes referred to as models with pharmacological isomorphism (9). Since psychopharmacological treatments of autism are largely symptom-oriented, animal models that mimic behavioral responses to clinical drugs are unlikely to give insight into the pathophysiology of the disorder (12).

The second class of animal models includes the animal models with face validity, where there is symptom similarity between a model and a disease. In case of childhood behavioral disorders, such behavioral isomorphism can be difficult to obtain. Many key symptoms of ASD are of a cognitive nature and thus difficult to model in animals. Even when autistic-like symptoms such as hyperactivity and stereotypic behaviors are modeled, it is unclear whether they arise from a specific autistic-like pathogenic process since stereotypy or hyperactivity can be induced by a great variety of drugs and manipulations (14).

The third group of animal models includes the animal models with construct validity. These models try to reproduce key pathogenic processes underlying the disorder. These models are based on the concept that behavioral deficits in humans are due to fundamental disturbances in the brain functioning that could be modeled in animals (12). Importantly, if successful, it is possible to model both a deficit (e.g., increased anxiety) and its pathogenic mechanisms (e.g., alterations in the limbic system) in the same setting. As far as autism is concerned, neurodevelopmental damage is believed to be the main pathogenic process leading to behavioral deficits (7,11,13). Thus, animal models of abnormal brain and behavior development are likely to give most insight into the pathophysiology of autistic-like abnormalities (11, 15).

The ASDs are believed to be caused by heterogeneous etiologic agents (e.g., genetic defects, drugs, viruses), therefore, multiple animal models are likely needed to fully mimic and evaluate the pathogenic processes (7, 11,13). Current animal model systems of developmental behavioral disorders utilize various gene-knockout approaches and numerous physical and chemical agents to derail normal brain development and produce behavioral deficits (16-19). There are very few animal models using viruses as teratogens, despite the fact that perinatal virus infections (e.g., herpes simplex, measles) induce CNS injury (1) and childhood behavioral disorders (13, 20, 21). Thus, there is the biological plausibility for using viruses as behavioral teratogens in animal model systems of autism (22-24). Virus infections associated with autism include rubella virus, cytomegalovirus, maternal influenza or mumps, postnatal mumps, congenital or childhood herpes simplex virus I and II infection, parvovirus B19 and HIV (5). Viruses may also produce autism indirectly, by inducing inflammatory responses in the brain, although classical signs of encephalitis (e.g., fever, malaise, and obtundation) are not necessarily associated with autism (5-7).

4. NEONATAL BORNA DISEASE VIRUS INFECTION AS A NEW ANIMAL MODEL OF AUTISM

Over the past ten years, we have developed and characterized the neuroanatomical, behavioral, neurochemical and immunological features of the first virus-induced developmental animal model of ASD: Developmental brain damage following non-inflammatory, persistent central nervous system (CNS) infection of neonatal rats with an RNA virus, Borna disease virus (BDV; refs. 25-36). The reliability of this model system have recently been confirmed and expanded upon by several other groups (37-42).

4.1. Borna disease virus

Although BDV represents the prototype member of a new class of viruses (*Bornaviridae*) in the

Mononegavirales Order, the BDV genome organization is similar to that of paramyxoviruses and rhabdoviruses (43-44). The BDV is an 8.9 kb, non-segmented, negative, single-stranded, enveloped RNA virus (44). The genome replicates in the nucleus, where the polyadenylated mRNA transcripts are synthesized and then transported to the cytoplasm (43,45). BDV particles are spherical, enveloped, and approximately 130 nm in diameter, with spikes of 7 nm in length and a nucleocapsid of 4 nm in width. Virions are released by budding from the cell surface (46).

The viral genome codes six open reading frames (ORF's; refs. 47-49). The ORF I encodes the nucleoprotein (p40); ORF II encodes the phosphoprotein (p24); ORF III encodes the matrix protein (p16); ORF IV encodes the glycoprotein (p56); and ORF V encodes the RNA-dependent RNA polymerase of BDV, the L-protein. Another ORF, ORF x 1, which overlaps with ORF II, and encodes the p10 protein, has recently been identified. All these virus-specific proteins have been detected in BDV-infected material, however, there are no detailed studies that define the functions of these BDV proteins (47-49).

The BDV is a natural pathogen in a wide range mammalian and avian species, from rhesus monkeys to ostriches (48). BDV is an extremely neurotropic virus and causes a persistent infection of neurons and astrocytes (48,50). Within the CNS, the limbic system (including the hippocampus), cerebellum, and the neocortex are major brain regions targeted by BDV (47-49). *In vitro*, BDV replicates persistently and noncytopathically, i.e. without killing infected cells (47-49).

4.2. Neonatal BDV infection

Infection of neonatal Lewis rats results in immunological "tolerance" to BDV, and these persistently-infected rats lack significant inflammatory cell response to the virus (51-52).

When compared to other experimental viral teratogens, neonatal BDV infection has a number of distinctive and unique advantages. Unlike many virus infections of the CNS that typically induce encephalitis with generalized brain damage and often death of the animals, in rats, neonatal BDV infection does not cause mortality or generalized brain damage, making detailed analysis of behavioral abnormalities possible (1-2).

Neonatal BDV infection-induced abnormal brain and behavior is not associated with T-cell immune-mediated response in the brain parenchyma (51-52). Neurodevelopmental damage induced by neonatal BDV infection is not mediated by a cell immune response or a direct lysis (25,29,53) by the virus as it occurs during neonatal infection with lymphocytic choriomeningitis virus (LCMV), rat parvovirus, or reovirus type III (54-56). BDV can replicate in the cell in such a way that the cell may survive, although there is a loss in cell function (48-49). Immunological clearance of BDV from the infected cell does not occur, and lifelong viral replication ensues (47-49). Hence, BDV infection allows us to study brain-behavioral relationship from a developmental standpoint in the setting of more naturalistic conditions than those produced by lesion or pharmacological manipulations (31).

The BDV infection may have direct relevance to human behavioral disorders (57). Seroepidemiological studies have shown an increased BDV seroprevalence in neuropsychiatric patients (47,58,59). Moreover, higher BDV RNA contents have been documented in peripheral blood mononuclear cells of neuropsychiatric patients than healthy donors (60-62). BDV antigen and RNA have also been detected in human brain samples collected at autopsy from individuals with a history of mental disorders (63-64).

Neonatal BDV infection is a unique teratogen since it allows us to analyze pathogenic mechanisms of behavioral disorders from different perspectives. On the one hand, neonatal BDV damage is characterized by distinct brain pathology and selective behavioral deficits, e.g., damage to the hippocampus may underlie deficits in emotional and cognitive domains (35). On the other hand, as a persistent infection, BDV produces metabolic alterations in selective neuronal populations without a gross injury (1,2). In this context, the BDV animal model gives us an opportunity for studying direct effects of the virus throughout entire life of the animal. Many of the features of disease pathogenesis and expression of developmental neuroanatomical and behavioral disease in neonatally BDV infected rats resemble ASD.

5. AUTISTIC-LIKE FEATURES OF NEONATAL BDV INFECTION

Developmental neuropathological abnormalities of individuals with ASD, and the mechanisms by which these abnormalities arise, are modeled by cerebellar/hippocampal developmental deficits in the neonatally BDV infected rats:

- key behavioral disturbances in sensorimotor, social, emotional and cognitive behaviors;
- developmental and regional abnormalities in central nervous system (CNS) neurochemical transmitters; and;
- contributions of the endogenous neuroimmune responses of the brain to neurodevelopmental brain damage.

5.1. Appearance, physical growth

Neonatally BDV-infected Lewis rats have normal physical appearance to the casual observer with no overt signs of CNS infection, such as malaise, fever, and anorexia (25). Neonatally BDV-infected rats have normal body shape and proportion but are overall much smaller than uninfected control pups (25,27). There are differences in the overall body size and weight, which appear to begin to emerge as early as at postnatal day (PND) 4 (27, 36, 37). The basis for runting is unclear since levels of glucose, growth hormone, and insuline-like growth factor-1 are normal (27). The amount of food ingested is similar in BDV-infected and control rats although a heightened taste preference for salt solution was documented in BDV-infected rats (27).

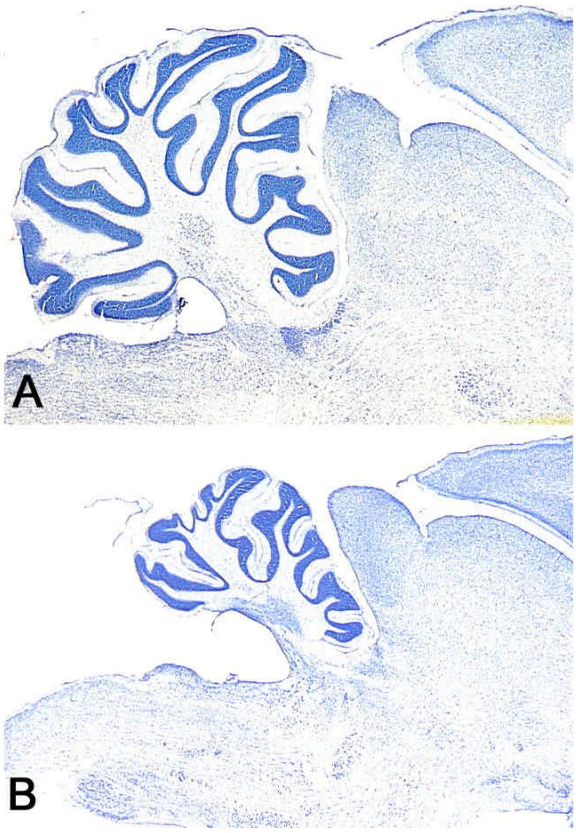

Figure 1. Representative sagittal section through the cerebellum at PND 30 of (A) sham inoculated Lewis rat, and (B) Lewis rat neonatally inoculated with BDV. Note severe cerebellar hypoplasia following neonatal BDV infection. Cresyl violet staining, original magnification x 12.5.

5.2. Neuropathology

5.2.1. The distribution of the virus

The general anatomical sites of infected neuronal cells is similar in neonatally and adult BDV-infected rats, with some notable exceptions (25, 37, 41). Soon after inoculation (PND 7-15), expression of BDV antigen is mainly localized to limited regions of the brain, i.e., neocortex, CA fields of the hippocampus, containing pyramidal neurons, and Purkinje cells of the cerebellum and thymus (25, 29, 37, 41). In contrast, the cerebellar granule cells seem to remain uninfected (28) and only few positive cells are present in striatum, nucleus accumbens or thalamus (41). Rare astrocytes, ependymal cells and oligodendrocytes appear infected by PND 14 (41). By PND 21, BDV strongly expressed in virtually all brain areas (39), indicating a diffuse involvement of the nervous system. Viral antigen can be detected within the cell bodies (i.e., cytoplasm) and high levels of viral proteins are readily detected by immunohistochemistry in the neuropil and peripheral nerve axons (25,39). The overall anti-BDV staining intensity appears to gradually decline after PND 180 (41). Furthermore, in contrast to the BDV infection of adult rats, neonatal BDV infection spreads beyond the confines of the central and peripheral nervous system, e.g., the peripheral blood mononuclear cells, the bone marrow, thymus , urine, feces and saliva (65).

5.2.2. Neonatal BDV infection-induced autistic-like developmental brain injury

Although data from histopathological and imaging studies do not always coincide, autopsy and imaging findings have largely supported the theory of a developmental etiology of autism (7, 66, 67). The major brain areas implicated in autism are the hippocampus, cerebellum, amygdala, frontal lobes, basal ganglia and brain stem (7, 67). Interestingly, BDV-induced developmental brain damage also includes the cerebellum, hippocampus, and neocortex.

5.2.2.1 Cerebellum

The cerebellum has an organized and laminar neuroanatomical structure that undergoes significant postnatal development in humans and rats (16). Decrease in the number of Purkinje cells is one of the more consistent findings in pathological studies of brains from autistic individuals (66,67). BDV-induced neurodevelopmental damage to the cerebellum is characterized by a specific time course following intracerebral inoculation with BDV of neonatal rats. Carbone and her colleagues have shown that the cerebella of BDV-infected rats develop normally until the end of the first postnatal week (28). By PND 14, the cerebella of BDV-infected rats showed evidence of arrested development, stunted size, decreased foliation, thinned and irregular internal granule cells layer and molecular layer. By PND 21 (i.e., the completion of the cerebellum development), the cerebellum in BDV-infected rats is significantly smaller than in control rats, with a profound size reduction in the cerebellar molecular layer and/or internal granule cell layer (ref. 28 and figure 1).

The BDV infects Purkinje cells by PND 7, but does not appear to infect cerebellar granule cells (28). Although Purkinje cells (PC) initially survive the BDV infection, starting at PND 27-30, many BDV-infected PCs are gradually lost an unknown mechanism (38, 40). As visualized with immunohistochemistry for PC-specific antigen, calbindin, there are numerous gaps within the PC layer and in the molecular layer that contains the PC dendritic trees. Although the granule cells in the external germinal layer and internal granule cell layer do not seem to be susceptible to infection by BDV *in vivo* or *in vitro*, granule cells are lost during the first PND 14-30 (28).

A qualitative review of brain sections from BDV-infected rats has suggested that BDV-associated cerebellar injury is not uniform but varies within the cerebellum. In BDV-infected rats, the distribution of immunoreactive PCs is somewhat variable. Some lobules appear to contain a fairly uniform complement of PCs whereas other lobules appear to be mildly or severely deficient in PCs (38, 40). The areas with the most severe granule cell loss appear to coincide with the regions where a high proportion of PCs is infected (40).

Apoptosis, or programmed cell death, is a mechanism in which cells undergo chromosome

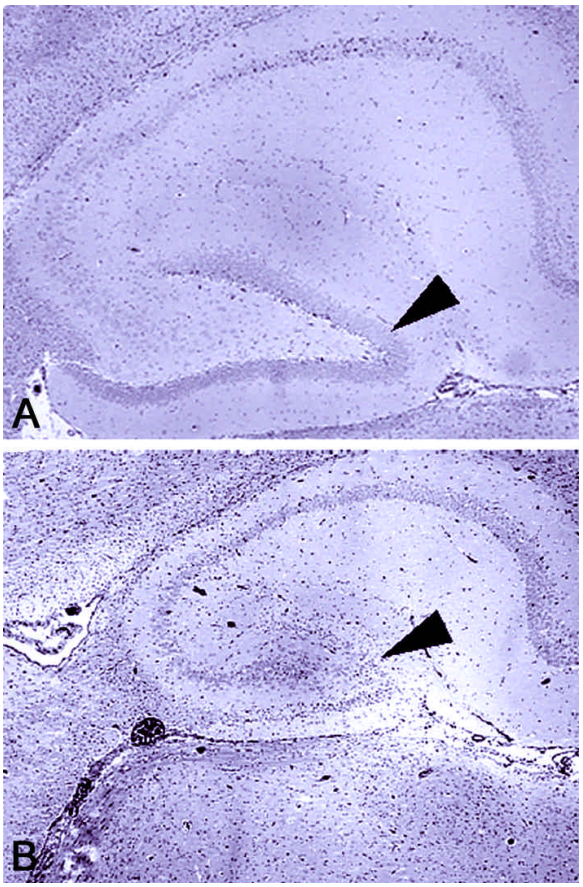

Figure 2. Representative sagittal section through the hippocampal formation showing the dentate gyrus (arrowhead) at PND 30 of (A) sham inoculated Lewis rat, and (B) Lewis rat neonatally inoculated with BDV. Note the dentate gyrus degeneration following neonatal BDV infection. Cresyl violet staining, original magnification x 40.

condensation, DNA degradation, and morphological changes in the nuclear membrane (68) Apoptosis plays an important role in CNS development and response to neuronal injury (69). It is possible that abnormal regulation of apoptosis, either failure of normal apoptotic sequences to proceed or excessive activity, may contribute to abnormal CNS architecture in neonatal infection with BDV (37). Apoptosis of the Purkinje cells has not yet been unequivocally demonstrated. In one study, terminal deoxynucleotidyl transferase dUTP-biotin nick end labeling (TUNEL) assays on paraffin embedded sections showed no indication of apoptotic cells in the PC layer at any time point examined (40). In contrast, another investigation found TUNEL-labeled cells in the PC layer between PND 28 and 42 (41). In the cerebellar granule cell layers, the number of apoptotic cells was found to be highest at PND 8. Between PND 14-48, apoptotic cells numbers in the granule cells layer were found to be between 2-6-fold higher in BDV-infected rats compared to uninfected controls (37, 40). Of note, the time discrepancy between apoptosis of granule cells and a loss of Purkinje cells might indicate that even if granule cell loss, already seen at PND 8, is unlikely to be due to loss of Purkinje cell, the virus replication in PCs and/or Bergman glial cells could lead to the disappearance of the external granule cells layer by affecting maturation and/or migration processes in the developing cerebellum (28).

5.2.2.2. Hippocampus

Abnormalities in the hippocampus and other structures of the limbic system have been described in autism (66,67,70). These abnormalities include reduced neuronal cell size, increased cell-packing density, and decreased complexity and extent of the dendrite arborization (66).

Neonatal BDV infection produces dramatic developmental damage to the hippocampus. Similar to the time course of the cerebellar damage, no overt pathology was found until PND 14. By the end of the third postnatal week, a reduction in the number of dentate gyrus neurons can be already noted in BDV-infected rats (ref. 30 and figure 2). By the end of the second month of life, the dentate gyrus is virtually replaced by reactive glial cells in BDV-infected rats (25, 30, 37, 39). Among replacing glial cells, microglial cells are thought to prevail over astrocytes (41). Qualitative evaluation of neurons in the CA1-4 regions of the hippocampus showed less dramatic, but clear, deterioration of cell density (25).

Similar to apoptotic processes in the cerebellum, apoptotic neurons were found in the dentate gyrus in BDV-infected rats as early as at PND 21 (41). In the hippocampus, apoptosis peaks at PND 28-30, and thereafter, the number of apoptotic neurons gradually decreases since activated glial cells begin to replace dying neurons (25, 30, 41). BDV-induced cell loss in the hippocampus is accompanied by a significant decrease in immunostaining for two synaptic markers, growth-associated protein (GAP-43) and synaptophysin (42).

5.2.2.3. Neocortex

In contrast to the cerebellum and hippocampus, BDV-induced damage to the neocortex has been studied in much less detail. By PND 45, BDV-infected animals exhibited a cortical shrinkage by about 30% (42). A reported selective loss of cells with diameter of greater than 100μm could account for the cortical parenchyma thinning (42). Additionally, apoptosis of pyramidal neurons in the neocortex of BDV-infected rats may also contribute to BDV-induced cortical shrinkage (41). Similar to the hippocampus, BDV decreased immunoreactivity for GAP-43 and synaptophysin is seen in the neocortex of neonatally BDV-infected rats (42).

5.2.2.4. Other brain regions involved

There are few data addressing whether a significant BDV-associated cell loss occurs in other brain regions, despite the fact that the virus seems to gradually infect cells all over the brain (25,39). Nevertheless, some neuronal loss appears to occur in deep cerebellar nucleus, ventral cochlear nucleus, and superior colliculus (41). These lesions were first visible at PND 21, peaked at PND 28-30 and continued through PND 42 (41). More rigorous

quantitative studies would be necessary to confirm reported qualitative evaluations.

5.2.2.5. The molecular mechanisms of neuronal loss

The molecular mechanisms responsible for BDV-associated apoptosis remain largely obscure. Available data point to BDV-induced alterations in the balance between the expression of pro- and anti-apoptotic genes, and a decrease in the expression of neurotrophic factors. For example, alterations in transcripts encoding genes associated with regulation of apoptosis appeared at PND 14 and persisted through PND 84 (37). While levels of mRNAs for FAS and caspase-1, two promoters of apoptosis, were increased throughout the brain from PND 28, mRNA for bcl-x(L), a neuroprotective factor that inhibits apoptosis, was decreased only in the hippocampus and cerebellum at PND 14-28, and PND 42, respectively. Levels of mRNA for caspase-3 or bax, pro-apoptotic factors remained unaltered by neonatal BDV infection (37).

BDV-associated reduction in expression of neurotrophic factors could also account for enhanced vulnerability of neurons in BDV-infected brains. For example, mRNA expression levels of neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) were significantly decreased after PND 14 in the hippocampus of BDV-infected rats compared to control animals (40). Similarly, levels of neurotrophins' receptors transcripts (e.g., trkC and trkB) were reduced in the hippocampus (both) and cerebellum (trkB) of BDV-infected animals at PND 21. Reduced trkC mRNA expression was confined to the dentate gyrus of the hippocampus, as assessed by *in situ* hybridization (40). No BDV-associated changes in levels of ciliary neurotrophic factor (CNTF), NT-4 and glial fibrillary neurotrophic factor (GFNF) were noted (37).

Billaud and colleagues have identified another possible factor that could be responsible for the induction of apoptosis following BDV infection. They found that BDV caused inhibition of glutamate uptake by feline primary cortical astrocytes (71). It is conceivable that impairment in the ability of astrocytes to uptake glutamate contributes to N-methyl-D-aspartic acid (NMDA)-mediated neurotoxicity and/or compromises the supply of substrate for energy production in neurons (71). It is unclear whether the virus directly damages the uptake processes in astrocytes or affects the astrocytes‘ functioning through excessive release of cytokines and chemokines secreted by neurons and other glial cells (paracrine mechanisms) or by astrocytes themselves (autocrine mechanisms) (33, 39).

Thus, neonatal BDV infection causes selective developmental damage to the cortex, hippocampus and cerebellum, i.e., the brain regions that continue to develop after birth, and, thus, that are susceptible to harmful effects of environmental insults (3,16). Apoptosis may be the major mechanism of neuronal death following BDV infection (37), although processes that induce apoptosis of neurons remain unclear. Both direct viral toxicity due to virus replication in a cell and indirect effects of multitude factors secreted by resident immune cells (e.g., cytokines, reactive oxygen intermediates) could be responsible for cell loss in BDV-infected rats.

5.3. BDV-induced immune alterations

Mounting evidence indicates that immune disturbances may contribute to the development of some cases of autism (72-77). In the developing child, genetically determined immune deficiencies could increase the risk for autism in two ways: 1) a pathogen (e.g., virus) or toxins might damage the developing brain; or 2) the pathogen might trigger an autoimmune mechanism that would interfere with brain functioning. In the mother, immune deficiency might allow a pathogen to persist *in utero*, damaging the fetal brain directly or triggering a maternal immune response that creates pathogenesis of the fetal brain (73). As our knowledge of the interactions of the immune, nervous and endocrine systems progress, complex links with the origin and course of psychopathology of childhood behavioral disorders are revealed (72). Neuroimmune disturbances and associated behavioral abnormalities caused by neonatal BDV infection provide a good animal model system for exploring the causal connection between neuroimmune alterations and psychopathological phenomena in autism.

In neonatal BDV model of ASD, there is no significant cellular inflammatory response following neonatal BDV infection (25, 37, 39, 41), a phenomenon mostly attributed to the immaturity of the rat immune system (51-52). The humoral immune response to the virus in neonatally-infected rats has been reported to be restricted, with anti-BDV antibody titers remaining below 1:10 until PND 133, and reaching only 1:100 by PND 300 (25). The precise mechanisms of immunologic tolerance in neonatally BDV-infected rats have not been conclusively demonstrated. In experiments using parabiosis and bone marrow transplantation, the defect in immune responses to the BDV in neonatally BDV-infected rats was linked to events that occurred during maturation of the immune cells, i.e., after exiting the bone marrow (25). Early spread of the BDV to the thymus leading to development of classical immune tolerance is suggested to be a potential mechanism to explain the absence of a BDV-specific cellular immune response (25).

Despite the restricted T-cell-mediated and B-cell-mediated immune responses in neonatally BDV-infected rats, a severe gliosis in various brain regions has been reported by many groups (25, 37, 40), implicating "endogenous" neuroimmune processes. Neural cells have been shown to be able to initiate, regulate and sustain an immune response, when different pathogens (viruses, apoptotic cells and cell debris) are recognized by glial cells via pattern recognition receptors (e.g., CD14 or scavenger receptor) (78-79). Activated glial cells can initiate an inflammatory cascade leading to the production of inflammatory and regulatory cytokines and chemokines that, in turn, are capable of producing an array of pathological changes, from moderately disturbed chemical neurotransmission to severe neuronal loss (78-79). Thus, BDV-activated glial cells may be one of the major pathogenic links to neurodevelopmental damage following neonatal virus infection.

5.3.1. BDV-associated activation of astroglia and microglia

Although infection of the newborn rat within 48 hours of birth leads to persistent infection without evidence of significant immune cell inflammation, evidence of endogenous neuroimmune responses by microglia and astrocytes are demonstrable by immunohistochemistry (25, 37, 41). There might be some regional specificity in the timing of the glial cells activation in the BDV-infected brain. For example, BDV-associated astrocytosis was evident in the Bergman glial cell processes in the cerebellum as early as at PND 3 (28). In contrast, no differences in glial fibrillary acidic protein (GFAP) staining and GFAP RNA expression was seen in the hippocampus between BDV-infected and control rats at PND 8 (39). Starting at PND 22, a sustained up-regulation of GFAP RNA expression was detected in the cortex, hippocampus and cerebellum, with the highest expression of GFAP RNA being reached by PND 33 (39). Of note, the strongest GFAP staining was seen in the dentate gyrus of the hippocampus where activated glial cells replace neurons of the dentate gyrus (25, 37, 39).

In addition to activated glial cells, mononuclear inflammation in leptomeninges, perivascular spaces and neuropil has been noted in the cingulate, frontal and parietal cortexes in BDV-infected rats (41). Immunohistochemical characterization of infiltrates revealed the majority of T cells (as was reflected by staining with antibodies to CD5 or the a/ß T cell receptor), few NK cells, monocytes/macrophages, perivascular cells, and B cells (3.2.3; OX42; TLD 1F5; and anti-rat IgG staining, respectively). Overall, number of CD4-positive and CD8-positive cells were similar, however, CD4-positive cells were present primarily in the meninges and perivascular spaces, whereas CD8-positive cells were diffusely distributed in the neuropil (37, 41).

In order to further characterize immune cells infiltrate, immunostaining with monoclonal antibodies OX-34 that recognize the rat immunoglobulin superfamily adhesion molecule CD2 (LFA-2) was performed on brain sections from neonatally BDV-infected rats (39). In the rat, CD2 is expressed on thymocytes, and tissue macrophages, but not on B cells (39). At PND 22 and 33, immunostaining with antibodies OX-34 revealed mild perivascular infiltrates and sporadic perivascular cuffing in the cortex and cerebellum of neonatally BDV-infected rats (39). After PND 48, perivascular cuffing was observed only very rarely. Throughout the observation period, the number of single CD2-positive cells in the brain parenchyma were slightly higher in BDV-infected rats than in control rats (39).

The activation status of the microglia in BDV-infected rats was also evaluated with monoclonal antibodies to the ED1 glycoprotein that predominantly expresses on the lysosomal membrane of the majority of tissue macrophages (39). In BDV infected rats, ED1 immunoreactivity was highest between PND 22 and 33, subsequently decreasing after PND 33 in the neocortex and cerebellum, and after PND 48 in the hippocampus. However, significant numbers of ED1-positive cells were detected in these three brain regions until PND 135. The majority of ED1-positive cells had morphological features characteristic of microglia cells. Cells with stellate morphology and long and thin, dendritic processes ("resting microglia") represented the largest population of ED1-positive cells (39). These cells were observed at all time in all brain regions examined. Round and rod shape ED1-positive cells with only few thick processes ("activated microglia") were also observed in all brain regions in BDV-infected animals. Their number peaked at PND 22 and 33 and decreased thereafter. These cells appeared to be more numerous in the cerebellum and the hippocampus than the cortex, especially at later time points. ED1-positive cells with more round and ovoid morphology and w/o processes, indicative of phagocytically active microglia/macrophages constituted the smallest ED1-positive cells population (39).

Using other histochemical markers of the glial cell activation, marked elevation of CD-4 expression on the majority of activated microglial cells has been demonstrated (37). In BDV–infected rats, OX-8-activated microglia were present in cingulate and retrosplenial cortexes, the dentate gyrus, and the molecular layer of the cerebellum at PND 28 and onwards. OX-8-positive microglia were also stained with antibody specific for CD-8ß, confirming the presence of the ß chain of the CD-8 molecule on activated microglia. After PND 42, CD-8 expression was limited to cells in the cerebellar molecular layer and was not detected at all after PND 168 (37,41). Immunoreactivity of iNOS was found only in CTX of rats at PND 28-35 and localized to single macrophages in neuropil, adjacent to perivascular cuffs (41).

The major histocompatibility complex (MHC) antigens are necessary for antigen presentation to T lymphocytes but are not usually expressed in normal brain tissue (1-2), however species- and strain-specific differences in MHC antigens expression have been described in intact animals (2). Earlier findings did not reveal significantly elevated expression of MHC class I and II molecules in the neural cells of neonatally BDV-infected rats (25). Recent studies demonstrated that by the end of fourth postnatal week, MHC class I was detected at higher levels in endothelial cells of neonatally BDV-infected rats (41). BDV rats expressed MHC class I on microglia and rare astrocytes and neurons. MHC class I expression peaked between PND 28 and 42, and decreased thereafter. Similarly, MHC class II expression levels and distribution of MHC class II were higher in BDV-infected rats at PND 28, and the expression was observed on microglia and inflammatory cells. Microglial MHC class II expression decreased rapidly thereafter and was difficult to detect after PND 42 (41).

Similar to MHC class I and II antigens, the cell adhesion molecule, e.g., PECAM-1, was expressed continuously on endothelial cells (41). After PND 28, levels of another cell adhesion molecule, ICAM-1, were also increased on endothelial and other vascular and perivascular cells of BDV-infected rats, with most

prominent staining being observed in the neocortex, hippocampus and cerebellum (41). Thereafter, ICAM-1 expression decreased.

Thus, available data suggest that a transient inflammation may occur in brains of neonatally BDV-infected rats. Although a temporal appearance of inflammatory infiltrates coincided with the peak of neuronal apoptosis, infiltrating cells are unlikely to play a major pathogenic role in neurodevelopmental damage by neonatal BDV infection (41). Instead, if BDV-induced influx of inflammatory cells do contribute to neurodevelopmental damage, their main participation might be restricted to an activation of resident cells of the brain immune system, e.g., microglia and astrocytes that, in turn, release a multitude of biologically active compounds (78-79).

5.3.2. BDV-induced activation of cytokines and chemokines

Reactive astrocytes and activated microglia are the main source of the production of cytokine and chemokine in the CNS (80). Recently, several groups have shown BDV-associated increases in levels of regulatory and pro-inflammatory cytokines and chemokines in neonatally BDV-infected rats.

The BDV up-regulates interleukin-1 ß (IL-1 ß), tumor necrosis factor-a (TNF-a) and transforming growth factor-ß (TGF-ß) mRNA levels in parieto-frontal cortex, hippocampus, hypothalamus and cerebellum at PND 7 and 28 (33). The cerebellar samples from BDV-infected rats exhibit the highest levels of IL-1ß, IL-1 receptor type I, IL-1 receptor accessory protein II, IL-1 receptor antagonist and TNF-a mRNA expression. Cytokine mRNA's are differentially up-regulated among brain regions and throughout the postnatal period.

A chronic up-regulation of IL-6; TNF-a; IL-1alpha, and IL-1ß has been reported in the hippocampus and cerebellum of BDV-infected rats (39). The increased expression was first observed by PND 7, and was maintained until PND 120-135. In contrast, in the frontal cortex, the expression was higher at PND 22 and 33, and declined by normal level at PND 75 (39). Others have demonstrated BDV-increased levels of transcripts corresponding to mRNA for IL-6 were in the majority of brain regions beginning at PND 28 (37). In contrast, levels of mRNA's for pro-inflammatory cytokines IL-2, IL-3 and interferon-? (IFN-?), and T-helper2 (Th2)-type cytokines, IL-4, IL-5 and IL-10, were similar in BDV and NL rats at all time points (37, 41).

There is a sustained expression of IP-10 and RANTES chemokines genes in neonatally BDV-infected rats (81). The marked increase in chemokine gene expression was seen already at PND 8. Consistently with previous reports (25), *in situ* hybridization studies confirmed that astrocytes were the major source for IP-10 expression (81).

Thus, a profound and chronic increase in levels of cytokines and chemokines is associated with neonatal BDV infection. In this context, it is tempting to speculate that those biologically active substances might have neurotoxic effects on neuronal metabolism and cause cell loss or alterations of chemical neurotransmission with resultant behavioral deficits. Of note, various effects of cytokines on the brain chemistry, the endocrine system and behavioral responses have been reported (82).

5.4. Behavioral deficits

BDV-induced neuroanatomical damage is likely to underlie the behavioral abnormalities observed in infected rats. Among BDV-associated behavioral deficits are selectively deficient social behaviors (decreased play solicitation and responses) (31); changes in emotional behavior (increased anxiety to changes in environment) (32); selectively reduced cognitive abilities (deficits in spatial memory and learning/contextual fear conditioning with normal cue-dependent fear conditioning) (30,32); and spontaneous locomotor activity (circadian rhythm and sleep cycle disturbances, hyperactivity) (27,37,53).

5.4.1. Sensorimotor behaviors

Although autistic disorders are heterogeneous in its manifestation, there is a subgroup of individuals with autism who display movements that appear to be unique for the disorder. In young children, common findings include increased joint laxity and hypotonia, clumsiness, apraxia, and toe walking (6, 83). A variety of stereotypic movements such as hand flapping, pacing, spinning, running in circles and self-injuring behaviors may be pathognomic of autism (6). Although the neurological substrate for autistic abnormalities remains unclear, the cerebellar pathology could underlie some movement disorders observed in autistic patients (67).

In the rat, cerebellum-controlled behaviors have a discrete, organized pattern of evolution, paralleling the physical development of the cerebellum: e.g., the ability to maintain static quadruped posture precedes the ability to maintain quadruped posture during movement, which is followed by the acquisition of simple motor skills (84).

Neonatally BDV-infected rats show no evidence of gross ataxia and have normal swimming speeds despite the significant cerebellar lesions (27). However, there is evidence of behavioral deficits that could be linked to developmental cerebellar damage. For example, locomotor hyperactivity, hyper-reactivity and stereotypy along with mild gait ataxia, mild hind paw spasticity and a deficient ability to hang on the dowel have been described in neonatally BDV-infected rats when tested as adult, i.e., 12-76 weeks of age (27, 37, 53). Recently, a developmental time course of sensorimotor deficits in developing BDV-infected rats (PNDs 4-30) has been described by testing proprioception, motor strength, and coordination skills (36). BDV-induced motor impairments were selective and correlated with the time course of BDV damage to cerebellar development. BDV-induced motor deficits were not seen until PND 14. By PND 21, BDV-infected rats had deficits in negative geotropism, fore and hind limb placing and grasping. BDV-infected rats also exhibited deficits in the ability to hold on to a bar and to cross a suspended bar.

Neonatal BDV infection significantly decreased the rat's responsiveness to the acoustic startle stimuli and attenuated habituation of the acoustic startle response (36). Notably, BDV-induced damage to the acoustic startle response was relatively selective, since prepulse inhibition of the acoustic startle remained unaltered in BDV-infected rats. Thus, BDV-induced abnormal development of the cerebellum is associated with selective impairments of sensorimotor and postural skills in Lewis rats.

5.4.2. Emotion and anxiety

Hypersensitivity to changes in the environment, and the ensuing anxiety and fear, are one of the hallmarks of the emotional disturbances in autism (5-7). Patients with autism show a wide range of emotional deficits, including inability to tolerate changes or transitions, reacting to imminent/recent changes with strong anxiety state (6).

Similar to autistic patients, neonatally BDV-infected rats exhibit a number of behavioral abnormalities that may be characteristic of elevated fear-elicited responses and an increased tendency to escaping when rats are exposed to novel/aversive stimulation (27, 32, 37). In a series of experiments, emotional abnormalities in neonatally BDV-infected adult Lewis rats were characterized by studying their species-specific fear-related responses (32). Compared to normal animals, BDV-infected rats exhibited locomotor hyperactivity and elevated defecation in a highly aversive, brightly lit open field. As expected, in a less aversive, dimly lit open field, uninfected controls increased ambulation whereas infected rats significantly decreased locomotor activity and defecation. BDV-infected rats also demonstrated increased sensitization of the startle response, suggesting a tendency toward elevated escape responses. Another evidence of BDV-associated greater anxiety has been recently reported for one-month-old BDV-infected rats, i.e., animals had prolonged behavioral inhibition upon introduction to the novel environment (for the first 30 minutes of testing). When rats were observed over subsequent intervals (30-60 and 60-90 minutes), young BDV-infected rats exhibited locomotor hyperactivity (37).

In summary, available data appear to indicate that neonatally BDV-infected rats are hyper-reactive to aversive and novel stimuli, possibly due to chronic emotional abnormalities. BDV-induced damage to the hippocampus and cerebellum may underlie the expression of elevated fear responses in rats (85).

5.4.3. Cognitive behavior

Mental retardation is commonly, but not invariably, associated with autism (6-7). However, based upon the sites of brain damage postulated for autistic patients (hippocampus and subcortical structures), it is not surprising that some autistic patients have characteristic abnormalities in specific types of memory, e.g., representational memory (i.e., related to higher order cognition and learning, and spatial relationships) (7, 11). The sparing of the neocortex in these individuals would be expected to leave habit memory intact (i.e., automatic connections between stimulus and response). This is consistent with the observation that anxiety can be induced in autistic children by even small changes in a stable, well-memorized environment, while, at the same time, some autistic children can perform impressive feats of rote memorization (7, 11). Notably, there are developmental differences in these types of memory, since habit memory develops before representational memory likely due to differences in maturation of various neuronal circuits for these two forms of memory (86).

The limbic system structures, such as those found abnormal in autism, are implicated in disturbances of learning and memory, including loss of recognition and associative visual memory (66, 86,87). Early damage to the limbic system might disturb acquisition and/or processing of novel information from daily life experiences; such a disruption might lead to autistic symptoms such as disordered cognition, social interactions and language deficits (70).

5.4.3.1. Spatial discrimination learning

One of the first behavioral studies of the neonatal BDV model included evaluations of spatial discrimination learning based on performance in the Y-maze and the hole board (53). In the simpler Y-maze, the rats were trained to distinguish between two symmetrical arms of a Y-maze where food was placed only at the end of the right arm. The BDV-infected rats made a significantly greater number of incorrect choices compared to uninfected control rats (53). For the hole board, a task designed to reflect an ability to process and remember spatial information, the rats were trained to search for food pellets placed diagonally in different holes. The mean number of errors in BDV-infected rats was significantly higher than in uninfected animals, and BDV-infected rats required significantly longer time to locate all baited holes (53).

5.4.3.2. Spatial learning and memory

Both the hippocampus and cerebellum play a major role in the acquisition of spatial navigation tasks, and lesions in these areas impair acquisition of a hidden platform location in the Morris water maze (MWM). MWM is a classic test for studying spatial learning and memory via navigation based on visual cues (88-89). The advantage of the MWM is the use of a swimming paradigm, rather than a running one, and, unlike running, swimming behaviors are minimally affected by cerebellar damage (89). In the MWM, neonatally BDV infected rats exhibited a performance deficit (30). At PND 72, the BDV-infected rats had difficulties in learning the location of the platform over a series of swim trials, as indicated by their failure to significantly reduce the time to find the submerged platform. Importantly, maximum swimming speed between infected and uninfected groups was not different (30).

5.4.3.3. Fear conditioning

Another behavioral task requiring the integrity of the limbic system, particularly the hippocampus, is contextual fear conditioning (87). In this task, rats exhibited species-specific fear responses to the context (e.g., the test chamber) previously paired with aversive stimuli (e.g.,

electrical foot shock or sudden loud noise). Freezing behavior (i.e., complete immobility) and defecation response can be used in rats for assessing the amount of contextual fear conditioning. Unlike untrained naïve animals, trained rats usually exhibit more freezing and/or defecation in the context previously paired with aversive stimulation, indicating that the association between the aversive stimulation and the situation where aversive stimuli were presented to a rat has established, and the memory about the aversive situation (i.e., context) has formed. Compared to control rats, BDV-infected animals demonstrated attenuated conditional freezing in the context previously paired with either sudden loud noise or foot shock, suggesting attenuated contextual conditioning. Interestingly, conditioned defecation response to the context was spared in BDV-infected rats, indicating that some components of the brain system mediating fear conditioning remained unaffected by the virus infection (32).

5.4.3.4. Aversive learning

Effects of neonatal BDV infection on aversive learning and memory were studied by assessing avoidance of pain (either taste aversion or shock). Compared to uninfected animals, neonatally BDV-infected rats demonstrated a significantly reduced inhibition of responses to aversive taste and or shock following the training procedure, indicating deficient learning and/or memory about past aversive experience (53). Thus, neonatally BDV-infected rats exhibit a great number of learning and memory abnormalities that seem to be due to developmental damage to the limbic system and the cerebellum following the virus infection.

5.4.3.5. Social behavior

Social deficits are a major behavioral abnormality in autism (6). Autistic children show a lack of attention to social stimuli, fail to achieve joint attention (wherein other individuals are "drawn into" the attention to the same object), display protoimperative gestures without protodeclarative gesturing, fail to imitate the parental actions, and have abnormal expression of social attachment (6-7). Play behavior is particularly abnormal in autism, and is characterized by lack of social engagement, and, instead, a tendency toward repetitive, stereotyped and nonfunctional object manipulation (5-7). Thus, deficits in social behaviors, and, specifically, play behaviors should be a characteristic feature of any animal model of autism.

Social deficits are also found in neonatally BDV-infected rats. Neonatal BDV infection induced abnormal social interaction and communication in Lewis rats when tested as old as 30-35 days of age (31). Studies were conducted using the resident/intruder paradigm. A resident rat was isolated for one week in order to increase social motivation (90). An unfamiliar rat (intruder) was placed in the resident's cage. This scenario is conducive to social interactions between the rats, often resulting in play behavior (measured as number of "pins," similar to a pin observed in a wrestling match). When play activity of the resident was analyzed, the control rat pairs exhibited significantly more pins than the pairs where either one rats or both rats were infected with BDV. One of the reasons for reduced play activity in the BDV-infected rats could be a decreased drive to engage their partner in social play. We used an observational paradigm to evaluate specific play soliciting behaviors (e.g., pounce, crawl over/under and darting) and showed that the control resident rats demonstrated significantly more play solicitations than the BDV-infected resident rats, regardless of intruder's infection status, indicating normal play readiness on the part of the normal but not BDV-infected rats. The reduced play activity in BDV infected rats was not due to reduced locomotor activity or "non-play" social behavior (31). In fact, compared to control animals, non-play interaction was elevated in BDV-infected rats, suggesting that the entire organization of the timely expression of different types of social interaction was significantly disturbed by BDV infection. These data have interesting neurodevelopmental implications, suggesting that play activity and social non-play activity are under the control of different neural systems undergoing unequal maturation during postnatal life (90).

5.5. Neurochemical alterations

Despite over thirty years of research on the neurochemistry of autism, only a few replicated neurochemical abnormalities have been observed, largely from indirect (blood or cerebrospinal fluid) measurements or observations of drug actions and behavioral abnormalities (91). Abnormalities in monoamines (dopamine, norepinephrine and serotonin) and neuropeptides (opiods) have been implicated in autism (91-92). In humans, neurochemical abnormalities are identified from serum, or, less commonly, in cerebrospinal fluid measures. Unfortunately, the relationship of peripheral blood/CSF measurements of neurochemicals and their metabolites to the levels of neurotransmitters in the CNS parenchyma is not well documented.

In BDV-infected rats, behavioral autistic-like abnormalities may be the result of virus infection-induced alterations in postnatal maturation of ascending monoaminergic projections to the damaged brain regions. Tissue concentrations of norepinephrine (NE), dopamine (DA) and its metabolite, 3,4-dihydroxyphenol acetic acid (DOPAC), and serotonin (5-HT) and its metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA) have been assayed by means of high pressure liquid chromatography with electrochemical detection in frontal cortex, cerebellum, hippocampus, hypothalamus, and striatum of neonatally BDV-infected and sham-inoculated male Lewis rats at PND 8, 14, 21, 60 and 90 (34). Both NE and 5-HT concentrations were significantly affected by neonatal BDV infection. The cortical and cerebellar levels of NE and 5-HT were significantly greater in BDV-infected rats than control animals at PND 60 and 90. Tissue content of NE in hippocampus was unaffected. In hippocampus, neonatally BDV-infected rats had lower 5-HT levels at PND 8 and significantly elevated levels at PND 21 and onwards. Neither striatal levels of 5-HT nor hypothalamic levels of 5-HT and NE were affected by neonatal BDV infection, suggesting that the monoamine systems in the prenatally maturing brain regions are less sensitive to effects of

neonatal viral infection. 5-HIAA/5-HT ratio was not altered in BDV-infected rats indicating no changes in the serotonin turnover in the brain regions damaged by the virus. Neither DA nor DOPAC/DA ratio were affected by neonatal BDV infection in any of the brain regions examined. These data demonstrate significant and specific alterations in presynaptic monoaminergic systems in neonatally BDV-infected rats.

Thus, neonatal BDV-induced neurochemical alterations appear to be in a line with observed behavioral deficits and, may suggest that BDV-induced developmental disturbances in the 5-HT brain system play a significant role in behavioral abnormalities. Even if the exact nature of the 5-HT changes in BDV-infected rats is still poorly understood, the ameliorative effects of some 5-HT compounds deserve further validation and may indicate the utility of the BDV model for screening new pharmacological treatments.

6. PERSPECTIVES

Neonatal BDV infection, as a new animal model of autism, has shown etiological, neuroanatomical, neurochemical and behavioral consistencies with different symptoms and syndromes of autistic spectrum disorders. The BDV model of autism seems to fall in the category of the animal models with both face and construct validities. Notably, behavioral autistic-like abnormalities demonstrated by BDV-infected rats (the behavioral isomorphism) are modeled using an etiologically relevant teratogen that induces neurodevelopmental damage and results in autistic-like deficits. Thus, BDV-induced neurodevelopmental damage is similar to the dysregulation of the developmental programs and associated behavioral disorders in autism (5-7). The BDV model uniquely combines such features as systemic brain abnormalities (e.g., immune and neurotransmitter disturbances) and selective damage (e.g., brain pathology and behavioral deficits), potentially being able to mimic a variety of putative pathogenic processes in ASDs.

The BDV model allows us to begin to test several pathogenesis hypotheses for autism. For example, our current investigations are aimed at characterization of effects of different genetic backgrounds on perinatal brain injury and resultant behavioral abnormalities. Developmental behavioral disorders (e.g., autism spectrum disorders) are heterogeneous conditions (7), and the interaction between genetic backgrounds and environmental factors is thought to contribute to the variability in disease outcomes and responses to different pharmacological agents (93-94).

Another aspect of genetic background effects on BDV-induced developmental damage could include a detailed examination of putative differences between female and male rats. For example, our pilot studies indicated that female rats appeared to be more severe damaged by the neonatal BDV infection compared to male rats. If so, a role of sex hormones in shaping BDV-associated damage also deserves future investigations.

A predictive potential of the BDV model could be addressed in more details by evaluating behavioral responses to clinically efficient pharmacological treatments. If successful, this line of investigations would stimulate the use of the BDV model in a broader "preclinical" paradigm for searching new treatments for ASD (95-96).

Future studies of the BDV models could be also aimed at further elucidating effects of the virus on molecular processes inside neuron and glial cell. For example, our preliminary data suggested that the virus replication in neurons of the raphe nucleus and/or the locus coeruleus might lead to alterations in the neurotransmitter systems, and, as a result, to behavioral abnormalities. Both *in vivo* and *in vitro* experiments could be used for pinning down specific molecular events associated with the viral effects on the metabolism of a single cell.

In summary, a decade of investigation of neonatal BDV infection in the rat brain has indicated its utility as an animal model system for studying the pathogenesis of abnormal brain and behavior development. Future experiments may provide us with a better understanding of the mechanism by which an environmental insult derails the developmental programs and leads to behavioral deficits.

7. ACKNOWLEDGEMENT

The work was supported by the NIH grant RO1 MH 48948-08A1

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Key words: Animal Model, Autism, Borna, Brain, Development, Environment, Rat, Review

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