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Research report

Developmental brain injury associated with abnormal play behavior in neonatally Borna disease virus-infected Lewis rats: a model of autism

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Abstract

Play behavior, nonsocial exploratory activity, and nonplay social interaction were observed in male juvenile Lewis rats with brain developmental injury following neonatal infection with Borna disease virus (BDV). These behaviors were tested using the 'intruder-resident' paradigm, with social isolation of residents for six days prior to testing. Four experimental pairings of infected (BDV) and uninfected (NL) rats were studied as follows: NL–NL; NL–BDV; BDV–NL; and BDV–BDV (the first member is the resident, the second member is the intruder). Observation of social activities was carried out for 10 min on two consecutive days. Nonsocial exploratory activity (e.g. ambulation and rearing) was similar in BDV and NL residents. Duration of nonplay social investigation (e.g. sniffing, approach, and follow) was higher in BDV residents as compared to NL residents when tested on the first test day. On the second day, all rats showed similar level of nonplay social interaction. When confronted with NL intruders, NL residents exhibited significantly more play behavior compared to the NL–BDV, BDV–NL and BDV–BDV pairs, when play behavior was measured by the number of 'pins'. Moreover, irrespective of a type of intruder, NL residents demonstrated higher play soliciting behavior than BDV residents, indicating attenuated readiness to play in BDV-infected rats. The number of pins and play solicitations in BDV–NL pairs significantly increased over the two days of testing, while play activity in NL–BDV pairs declined on the second test day. This pattern suggests that the degree of social reinforcement on the first day of testing affected the level of play on the second day. These data demonstrate deficits in play behavior and other social interactions following BDV-associated developmental brain injury, thus supporting the value of the neonatally BDV-infected rat as an animal model of autism.

Keywords: Borna disease virus; Lewis rat; Social interaction; Play; Brain; Development

1. Introduction

Borna disease virus (BDV) is a negative strand, non-segmented RNA virus that is the prototypic member of Bornaviridae, a new class of virus in the Mononegavirales order, and is a human pathogen [26]. BDV inoculation into newborn Lewis rats causes a persistent infection of the brain, without an apparent cellular immune response, and the rats appear relatively normal

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to the casual observer without signs of encephalitis. However, careful study of neonatally BDV-infected rats has revealed significant developmental neuroanatomical and behavioral abnormalities, including loss of cerebellar neurons and emotional and cognitive deficits [4,5,9].

Interestingly, viral infections of the brain (e.g. rubella virus, herpes simplex virus, cytomegalovirus and human immunodeficiency virus) are one of the documented etiologic agents of autism, a common behavioral disorder affecting one in 1000 children [7]. Intrauterine infection with rubella virus is the best documented virus–autism etiologic link, with a large number of autism cases identified in children infected in utero during the rubella virus epidemic in New York City in the 1960s [6]. Since the cerebellum undergoes substantial pre and post-natal development in many mammals, it is not surprising that this area is particularly vulnerable to developmental damage during pregnancy and in the postnatal period. Autism is believed to stem from injury to normal brain development; not unexpectedly, the cerebellum is reported to be abnormal by histological and neuroimaging analysis in some cases of autism [8,11,21].

Although much is known about the clinical manifestations of neurodevelopmental disorders such as autism, our basic understanding of the neuroanatomical basis of the disorder and the potential relationships of particular behavioral pathologies to specific alterations in neural development remains largely unknown. Since there is a paucity of animal models for studying autism, and no model using viruses as injurious agents, we sought to explore the possibility that the neonatally BDV-infected rat model system may share some behavioral consistencies with autism, and, thus, possibly serve as a useful animal model system to provide insights into the pathogenesis of the defining symptoms in autism.

As a characteristic symptom of this disorder, autistic children show impoverished or atypical social behavior and fail to engage in social interaction, particularly cooperative play, with peers [12,27]. There is likely to be significant information relevant to human play derived from a study of social play behavior in the BDV rat model, since play behavior in all mammalian species appears to have similar characteristics. Importantly, whatever differences between human and animal play exist, there is a common theme on function(s) and goal(s) of play in children and maturing animals: in all mammals, social play helps develop social-affective skills while object play helps develop manipulative-cognitive skills [19,24].

In rats, play is characterized by chasing, sparring, and wrestling movements, and can be readily distinguished from adult behavioral counterparts by several criteria [20]. A flurry of play movements often terminates after one juvenile assumes a play-dominance stance over an inverted second juvenile, termed a 'pin' [19]. Peak play frequency occurs around 30–35 days of age [24], and coincides with the final maturation of the cerebellar cortex in normal animals [1,13]. The present work constitutes an evaluation of several kinds of social activity in juvenile rats infected at birth by BDV, with a particular emphasis on play behavior. Specifically, we sought to identify if the neurodevelopmental injury following BDV infection produced abnormalities in the development of exploratory activity, nonplay social interaction and play behavior compared to normal rats.

2. Material and methods

2.1. Subjects

Inbred Lewis pregnant rats (16–18 days gestation) were purchased (Harlan, Indianapolis, IN) for these studies. All rat pups were born and reared in the animal vivarium at CBER, FDA (Bethesda, MD). Rat pups were raised with their dams in $45 \times 26 \times 23$ cm^3 pan-type polypropylene cages with an overhead wire grid supporting food pellets and water bottle, and containing a 1–2 cm layer of wood-chip bedding. The infected animals were kept in a special isolation hood 'DUO-FLO' (Bio-Clean Lab Product, NJ). The sham-inoculated rats were kept in the same room. Rats were maintained on a 10:14 h light:dark cycle (lights on at 08.00) and had free access to food and water. Room temperature was maintained at approximately 21°C.

2.2. Inoculation

Each litter of rat pups was inoculated intracranially within 24 h of birth either with 0.02 cm^3 of CRP3 BDV strain or uninfected inoculum, as described previously [4]. The viral inoculum leads to 100% infection of the neonatal rats confirmed by gross evaluation of the brain [5]. Infected litters and uninfected control litters were housed separately. For behavioral experiments, no more than two animals from any litter served in a given experimental condition.

2.3. Procedure

Previous studies have shown that social isolation enhances motivation for social interaction [14,18]. Thus, on postnatal day 28, all animals were weaned and assigned to one of two conditions: individual housing or group housing (three rats per cage). The individually housed BDV-infected and sham-inoculated rat were considered 'resident' subjects and were isolated for 6 days prior to testing. The rats from the group cages were considered 'intruder' subjects. Both 'residents' and 'intruders' were housed and tested in the same type of the cage, as mentioned above. Pairs of infected (BDV)

and uninfected (NL) rats were studied as follows: NL–NL; NL–BDV; BDV–NL; and BDV–BDV (first member of the pair listed is the resident, second member is the intruder, e.g. NL (resident)–BDV (intruder) pair = NL–BDV). In this way, we were able to separately estimate effects of virus infection on the behavior of intruder and resident rats. Using this paradigm, there is no risk of virus spread from infected to uninfected rats during their brief interaction. BDV is extremely difficult to transmit from rat to rat by social contact, without direct inoculation, as demonstrated by the housing of infected rats with uninfected rats for several weeks without transmission of the infection (Carbone, unpublished data).

In accordance with the USDA biosafety rules, inoculations and behavioral tests were carried out in a biosafety cabinet ('NUAIRE', MN). The illumination in the cabinet was generated by a single luminescent lamp (100 W). Just before the beginning of testing, a cage with a resident and a cage of group housed intruders were placed in the cabinet for a 5 min acclimation period; afterwards, an intruder was placed into the resident's cage. Each pair of animals was tested in the resident's cage for 10 min per day for two consecutive days. The tests were carried out at 9–11 a.m. or at 4–6 p.m. One half of the rats was tested in the morning, and the other half was tested in the evening. Initially, one half of the residents was tested with intruders of the same viral status (infected or uninfected) for a 2-day experiment, then the same residents were re-tested with intruders of the other viral status for another 2-day experiment. The other half of the residents were tested in the opposite order. Thus, a single resident was confronted with both infected and uninfected intruders for four consecutive days. A single intruder was used once for each given pair. In total, 11 BDV residents and 10 NL residents, and 21 BDV intruders and 21 NL intruders were tested.

The behavior of rats was recorded on videotape and analyzed after the test sessions. The following behavior of both residents and intruders was assessed: (i) nonplay social investigation was defined as approaching/following, sniffing, nosing, or grooming of partner and the overall duration of all these behaviors was measured (passive contact such as sitting or lying with bodies in contact was not included in this social score); (ii) social play was measured as number of pins (recorded when one animal had its dorsal surface towards the ground with the other animal was above [19]); and (iii) play soliciting behavior was assessed by total number of (1) pounces, (2) crawls over/under, and (3) darts (i.e. rapid running movement by resident towards, parallel to, or away from intruder). Behaviors (i), (ii) and (iii) are believed to constitute play solicitations initiated by the resident to stimulate interactive social play [23]. Nonsocial exploratory activity was defined as the duration of ambulation around the cage and rearing on the hind limbs. After completion of the experiment, each animal was weighed.

3. Data analysis

The data were assessed with three-way mixed model analysis of variance (ANOVA), for the factors of viral status of the resident, viral status of the intruder and test day. Appropriate pair-wise comparisons were performed with a Tukey test. Acceptable statistical significance was established as $P < 0.05$.

4. Results

4.1. Weight

As previously reported, body weights of the BDV-infected rats (105.6 ± 2.1 g) were significantly ($P < 0.01$) less than those of the sham-inoculated animals (166.6 ± 2.8 g), when assessed on the last day of testing [4].

4.2. Nonsocial exploratory activity of residents

Fig. 1 depicts the mean duration of ambulation and rearing of the resident rats from each of the four experimental pairings. ANOVA revealed a significant effect of test day ($F(1, 67) = 5.43$, $P < 0.05$) while no main effects of the resident or intruder status were found (all $P > 0.05$), and there were no significant interactions.

4.3. Nonplay social interaction of residents

As shown in Fig. 2, BDV residents exhibited significantly more nonplay social exploration of intruders than NL residents ($F(1, 62) = 8.96$, $P = 0.004$). This difference was mainly seen on the first day of testing and disappeared on the second day, as demonstrated by a significant resident status × day interaction ($F(1, 62)$, $P = 0.023$). On the second day of testing, nonplay social activity of BDV residents decreased while nonplay social activity of NL residents increased slightly. However, a separate one-way ANOVA of the data for the second day indicated no differences between pairs, all $P < 0.05$. Additionally, a one-way repeated measure ANOVA did not reveal any significant changes in the time of social interaction for all the pairs over the two days of testing, with the slight increase in nonplay social activity for the NL–NL pairs being close to, but not reaching, statistical significance ($F(1, 15) = 3.7$, $P = 0.095$).

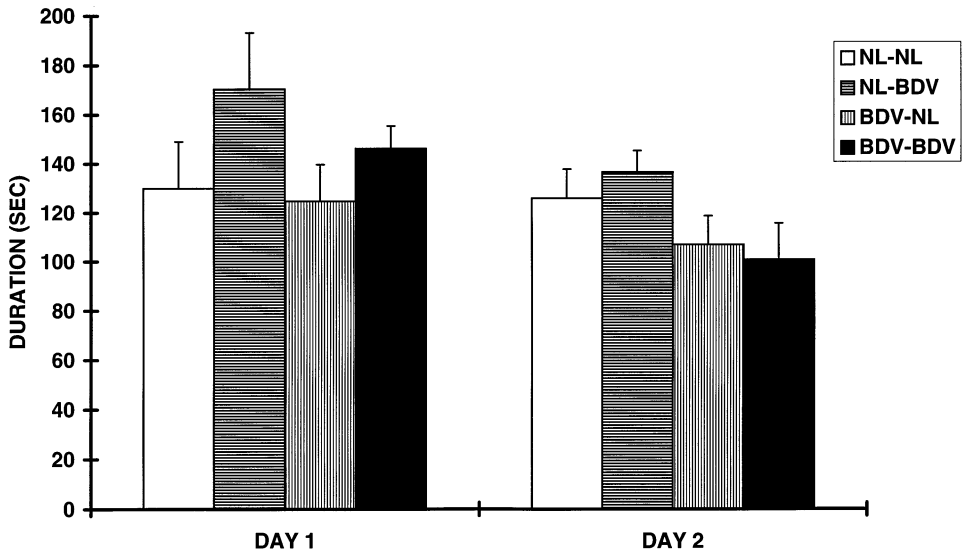

Fig. 1. Exploratory nonsocial activity in resident rats: sham-inoculated (NL–NL, NL–BDV) and BDV-infected (BDV–NL, BDV–BDV). Bars represents the means ± SEM for duration (s) of exploratory nonsocial activity (e.g. ambulation and rearing).

4.4. Play behavior

As demonstrated in Fig. 3, the inclusion of BDV-infected rats either in the resident or intruder role significantly reduced play behavior. ANOVA of the number of pins revealed significant effects of both the viral status of the resident ($F(1, 71) = 45.2$, $P < 0.001$) and the viral status of the intruder ($F(1, 71) = 18.6$, $P < 0.001$). BDV residents pinned intruders less frequently than NL residents, and BDV intruders were pinned less than NL intruders. There were also significant resident × intruder ($F(1, 71) = 36.2$, $P < 0.001$), resident × day ($F(1, 71) = 4.3$, $P = 0.042$), intruder × day ($F(1, 71) = 5.1$, $P = 0.027$) interactions. Post-hoc comparisons indicated: (i) overall, the number of pins was greater in the NL–NL pairs than in all other groups; (ii) that the number of pins by NL residents was higher on the first day of testing; and (iii) play behavior significantly increased on the second day in the BDV–NL pair, while there were no reliable changes over the 2 days of testing in the other groups.

4.5. Play solicitation

Fig. 4 shows the mean frequencies of play solicitation. NL residents displayed more soliciting behavior than BDV residents, as an ANOVA revealed a significant effect of viral status of the residents ($F(1, 60) = 37.82$, $P < 0.001$). There were no significant effects of the viral status of the intruder or day of testing, and there were no significant interactions. Post-hoc comparisons indicated that the infected residents in BDV–NL pairs demonstrated that the frequency of play solicitation increased from the first day to the second day tested. A separate analysis of the data for the BDV–NL pairs indicated that there was a significant increase in the number of solicitations by BDV residents on day 2 as compared to day 1 ($F = 2.95$, $P = 0.018$). In contrast, NL residents in NL–BDV pairs had a trend toward decreasing their soliciting activity on the second day, although the trend did not reach significance ($F(1, 14) = 0.26$, $P = 0.63$).

4.6. Nonsocial and social behaviors of intruder rats

Table 1 summarizes the data on nonsocial exploratory and nonplay social behaviors in intruder rats. ANOVA of the exploratory activity data from intruders revealed a significant effect of day ($F = 6.2$, $P < 0.05$), but no effect of viral status of the intruder or resident, and no significant interactions. There were no significant main effects in nonplay social interaction. However, there was a significant intruder viral status × resident viral status interaction ($F = 8.92$, $P < 0.01$) with higher nonplay social activity in BDV–NL, NL–BDV as well as in BDV–BDV pairs. Finally, there were no significant differences in the frequency of solicitations by intruder rats.

5. Discussion

We identified prima facie similarities in the neuroanatomy and the developmental nature of brain disease in children with autism and in neonatally BDV-infected rodents. Therefore, we tested rats infected with BDV at birth for evidence of hallmark symptoms of autism, abnormal social behavior; in particular, play activity and play solicitation [12,28]. As anticipated, neonatally BDV-infected Lewis rats

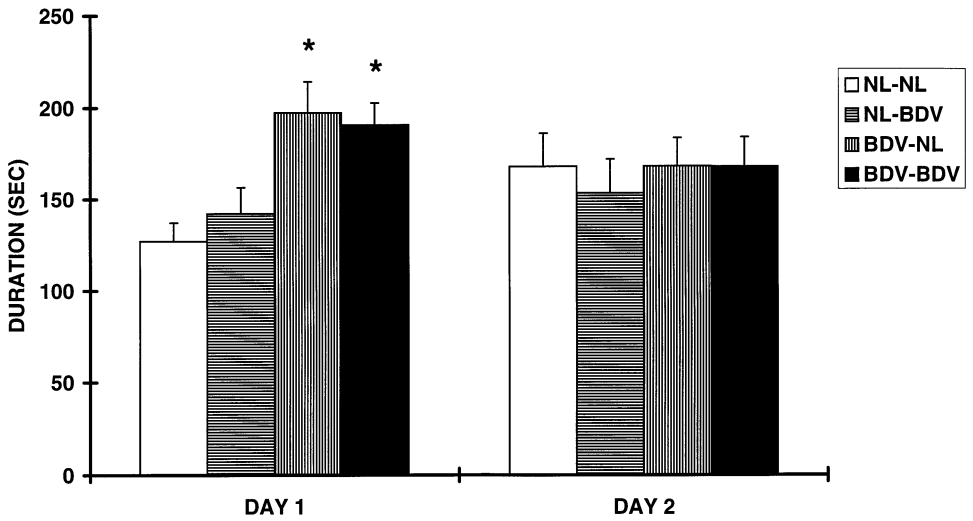

Fig. 2. Nonplay social activity in residents rats: sham-inoculated (NL–NL, NL–BDV) and BDV-infected (BDV–NL, BDV–BDV). Bars represent the means ± SEM for duration (s) of nonplay social activity (e.g. sniffing, nosing). $*P < 0.05$ compared to NL residents on the first day of testing.

demonstrated attenuated play behavior compared to sham-inoculated animals. This abnormal play behavior was not due to reduced locomotor or nonsocial exploratory activities because levels of ambulation and rearing were similar in infected and normal animals in our 10 min testing paradigm. In fact, previous work demonstrated that neonatally BDV-infected rats have increased spontaneous horizontal locomotor activity as compared to the uninfected control rats when tested over a 24 h period [4]. Moreover, the reduced play behavior was unlikely to be a result of an overall reduction in social interaction in infected rats, since our present data demonstrate that infected rats had a similar or even higher nonplay social activity than uninfected rats. Notably, normal to enhanced activity in BDV-infected rats is consistent with other cerebellar damage models, as evidenced by increased open-field activity in rats following the lesion to the vermis [22] and enhanced exploration in postnatally X-irradiated rats with depleted cerebellar granule cells [16], and abnormal behavioral inhibition in Purkinje cell degeneration mutant mice [15]. Taken together, these data suggest that the attenuation of play behavior following neonatal BDV infection was specific and not due to nonspecific decrease in motor activity.

The neuroanatomical basis of decreased play activity in the BDV-infected rats is unclear. Since the effects of cerebellar damage on play in rats have not been extensively studied [19,25], whether the current findings of decreased play are a result of the abnormal cerebellar development in BDV rats remains to be demonstrated. Since no reliable data are available about the viral damage to the other brain structures underlying play activity in rats (e.g. the cerebral cortex [25]), impairment of the organization of social play in neonatally BDV-infected rats due to the persistent viral brain infection of the other brain areas cannot be ruled out.

We measured resident play solicitation behavior to determine if the reduced play activity in BDV-infected rats was a result of a decreased readiness of the infected rat to be involved in play interaction. Notably, a reduction of play behavior may be effected by a decrease in play solicitation by the partner rats, as well as by a lower willingness of the partner to engage in play once it is solicited. Play solicitation or play signaling is a set of behaviors inviting social play or signaling readiness to engage in social play [23,24]. In the juvenile rat, specific behaviors classified as play soliciting include pouncing, tail-pulling, hair-pulling, crawling over/under, and darting [23]. Solicitation behaviors have been suggested to reflect appetitive phase of social play (the play motivation) [19,25]. When observed in pairs where one rat is isolated prior to testing, isolated resident rats initiate more play than the group-housed intruder rat. Play solicitation in resident rats also occurs largely irrespective of the intruder's willingness or capacity to be involved in play. For example, scopolamine blocks the ability of juvenile rats to engage in play behavior, yet scopolamine does not diminish the treated rat's attractiveness as a potential play partner to other juveniles. Thus, scopolamine-drugged rats are an effective social stimulus with which to induce solicitation behavior in an untreated partner [23]. We found that NL residents exhibited significantly more play solicitation than BDV residents, whether or not residents were paired with infected or uninfected intruders. Thus, BDV-infected rats' unwillingness to engage in play was not due to a lack of play solicitation behavior by their partners.

Play soliciting of a normal rat to a nonplayful stimulus (e.g. scopolamine-treated rat) resembles soliciting to a playful stimulus; however, in this case, the introduc-

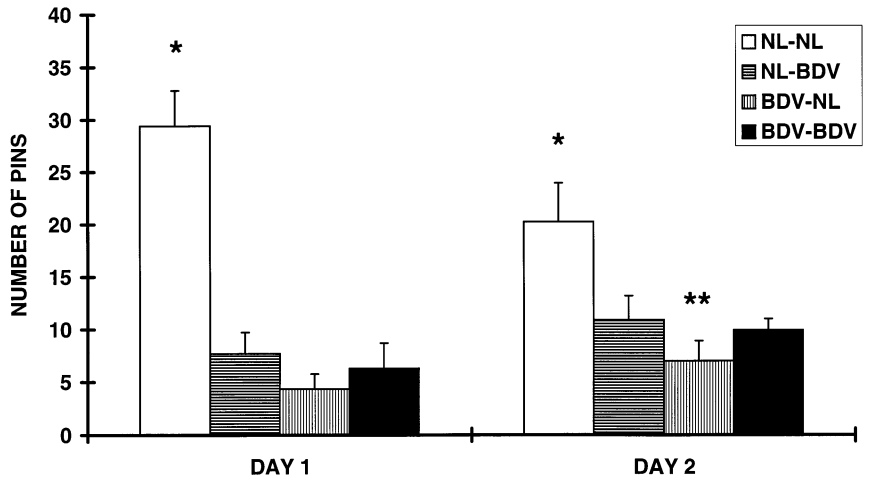

Fig. 3. Play activity in resident rats: sham-inoculated (NL–NL, NL–BDV) and BDV-infected (BDV–NL, BDV–BDV). Bars represent the means ± SEM for number of pins, as demonstrated by residents. *$P < 0.05$ compared to all other pairs; **$P < 0.05$ compared to day 1 for the same pair.

tory play solicitations do not merge into typical play behavior (e.g. pinning). Normally, the subject intermittently continues to solicit play until the lack of social reinforcement (i.e. play responses in the solicited, scopolamine-treated rat) result in extinction of play solicitation behavior. This extinction phenomenon may have played a role in our infected–uninfected mixed pairs, since the pattern of responding to play solicitations changed over the two day testing period. In BDV–NL pairs, both the frequency of play solicitations and the number of pins increased on the second test day as compared to the first test day. In contrast, NL–BDV pairs displayed a moderate decrease in the frequency of soliciting on the second test day. Both trends may reflect the influence of intruders' behavior on frequency of cooperative play in rats: (1) the NL intruder's repetitive play solicitations eventually facilitate play behavior in BDV residents, while (2) insufficient social play reinforcements from BDV intruders appears to lead ultimately to extinction of play solicitations by NL residents.

We analyzed the intruders' responses to determine the possible effects of intruders on social behavior during the pair interaction; for example, differences in social behavior observed in NL–BDV versus NL–NL pairings and BDV–NL versus BDV–BDV pairings might have resulted from a lower readiness of infected intruders to engage in social interaction. No differences in social activity of infected and normal intruder rats were found, although this result may be due to a 'floor effect', i.e. a low level of overall social activity in intruder rats. This low level of activity on the part of the intruders could be the result of the moderate aversive stimulation resulting from being tested in the strange resident rat's cage. It is possible that testing social and play behaviors of rats in 'an independent territory' paradigm would provide information on the potential role of the viral status of the intruder on the level of overall social behavior.

Differences in the number of pins in NL–BDV and BDV–NL pairs compared to NL–NL pairs might also have been the result of different weights of BDV-infected and uninfected rats. For example, in sex-mixed pairs, dominance asymmetries in play were found to be partially governed by weight differences [19,20]. However, the resident rats of the similarly sized BDV–BDV pairs also demonstrated fewer pins than normal residents of size-matched NL–NL pairs, suggesting that all of the differences noted in infected rat's play behavior cannot be solely ascribed to weight differences between rats.

The degree of illumination during testing can affect a rat's behavior. High levels of illumination increase anxiety, suppressing exploratory and social interactions, including play behavior [10,19]. Although it is possible that the reduction in play behavior in BDV residents was the result of increased sensitivity to the level of illumination, this seems unlikely. Increased sensitivity to light and, thus, increased anxiety would be expected to induce an decrease in overall activity relative to controls, while BDV resident rats showed a higher level of nonplay social interaction under these conditions. In addition, the level of illumination during testing did not exceed that under which our animals are normally housed, and rats lose their aversion to illumination following pre-test bright-light housing [10]. Finally, Dittrich et al. have suggested that neonatally BDV-infected rats are less anxious than normal rats when placed in an open-field test [9] and, in some cerebellar lesion (e.g. vermal lesion) models, rats are less affected by factors that suppress activity in normal animals, including level of illumination [22]. Thus, the conditions used in the

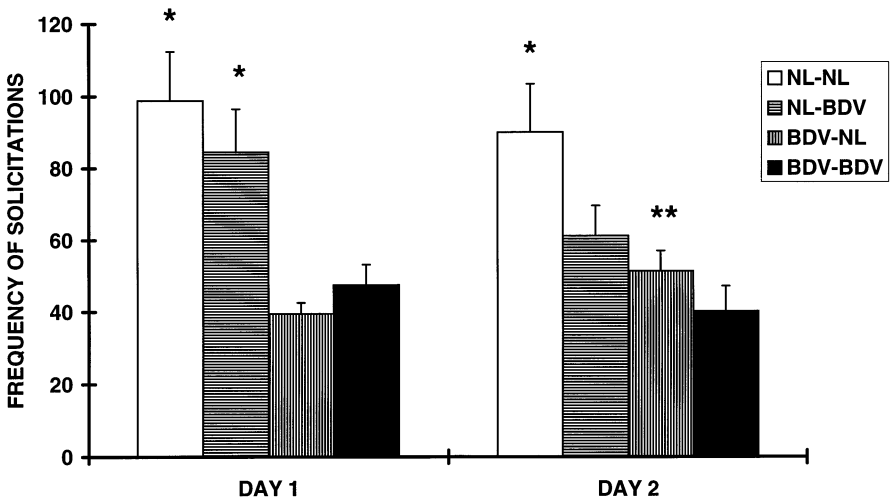

Fig. 4. Solicitation behavior in resident rats: sham-inoculated (NL–NL, NL–BDV) and BDV-infected (BDV–NL, BDV–BDV). Bars represent the mean ± SEM for frequency of solicitations. *$P < 0.05$ compared to BDV residents; **$P < 0.05$ compared to day 1 for the same pair.

present work for testing social behavior were unlikely to selectively affect play in the BDV-infected rats.

The present work is the first to describe abnormal social activity, particularly play behavior, in the neonatally BDV-infected rats. More importantly, it is the first report of evidence that improvement in attenuated play behavior is possible even in rats with profound neurodevelopmental and behavioral abnormalities. An increase of the frequency of play solicitations and the number of pins for the BDV–NL pairs over the 2-day testing period may suggest that some serious developmental abnormalities are amenable, at least in part, to recovery and that the recovery can be actively influenced by the subject's experiences.

Combined with the neuroanatomical abnormalities (e.g. cerebellum) and developmental pathogenesis of disease, our present findings of decreased play activity in BDV-infected rats provide additional support for the utility of neonatal BDV infection as an animal model for autism. The neonatally BDV-infected rat model shares a number of similarities with the syndrome of autism. For example, these infected animals, like autistic patients, have deficient information processing for an ongoing event and have deficits in learning and long-term memory [9,27] (Carbone unpublished data). Additionally, autistic children demonstrate a failure to appreciate the emotional significance of incoming stimuli [7,27]. In a similar fashion, BDV-infected rats fail to exhibit normal fear-related responses (e.g. freezing) when tested in the open-field [10]. Although there are few empirical studies on play in autistic children, it has been a common clinical observation that such children are especially deficient and aberrant in generating and elaborating social play [12,28]. Therefore, since one of the defining characteristics of autism has been a deficit in social interactions, the demonstration that rats with BDV infection have decreased levels of social play behavior provides an additional strong parallel between the pathogenesis and expression of neonatal BDV infection and autistic symptomatology.

Other behavioral animal models of autism have been reported. Recently, Bachevalier proposed a model based upon on neonatal lesioning of the amygdala and/or hippocampus in rhesus monkey. He found that monkeys with neonatal medial temporal lobe lesions showed numerous socioemotional and cognitive abnormalities [3]. Amaral et al. have reported that male rhesus monkeys raised in a natural setting and receiving bilateral ibotenic acid lesions of the amygdaloid complex have a dramatic tameness and varying degrees of altered temperament when tested in dyadic interactions [2]. Another model for addressing the pathogenic mechanisms of abnormal social behavior has been developed by Lijam et al. [17]. They found that mice lacking one of the three mouse homologues of the Drosophila segment polarity gene, 'Disheveled', exhibited reduced social interactions, including differences in whisker

Table 1
Nonsocial and social behaviors of intruder rats

Pairs		Exploration	Nonplay social activity	Solicitations
Resident–intruder		(s)	(s)	(frequency)
NL–NL	day 1	82 ± 16	38 ± 9	5 ± 1
	day 2	46 ± 12	8 ± 4	0 ± 0
BV–NL	day 1	70 ± 8	54 ± 11	1 ± 0
	day 2	60 ± 11	69 ± 16	4 ± 2
NL–BV	day 1	102 ± 23	65 ± 13	1 ± 0
	day 2	61 ± 17	62 ± 16	3 ± 1
BV–BV	day 1	76 ± 12	43 ± 9	4 ± 2
	day 2	62 ± 14	58 ± 14	5 ± 2

The data are presented as means ± SEM.

trimming, deficits in nest-building, less huddling and subordinate responses in a social dominance test. Based on the multifactorial etiology of autism (e.g. genetic, viral, pharmacological), a variety of animal models may have validity for the study of autism [7]. The ability of virus agents to induce autism, and the lack of a virus-induced model for autism, suggests the consideration of the neonatally BDV-infected rat model system for the study of autism.

Our findings in the rat model system and recent reports of BDV infection in humans begs the question as to whether BDV infection may directly lead to autism or autistic-like syndromes in children [26]. While our studies cannot determine whether BDV is a direct etiologic agent of autism, the results reported here demonstrate a number of similarities between the neonatally BDV-infected rat and children with autism. While confirmation or refutation of a role for BDV in direct induction of autism in humans is pending, this model remains useful for studying some of the underlying mechanisms in this significant developmental disorder.

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