Herpes Simplex Virus-2 Variation Contributes to Neurovirulence During Neonatal Infection

Herpes simplex virus (HSV) infection of the neonatal brain causes severe encephalitis and permanent neurologic deficits. However, infants infected with HSV at the time of birth follow varied clinical courses, with approximately half of infants experiencing only external infection of the skin rather than invasive neurologic disease. Understanding the cause of these divergent outcomes is essential to developing neuroprotective strategies. To directly assess the contribution of viral variation to neurovirulence, independent of human host factors, we evaluated clinical HSV isolates from neonates with different neurologic outcomes in neurologically relevant in vitro and in vivo models. We found that isolates taken from neonates with encephalitis are more neurologically relevant in vitro and in vivo models. These findings suggest that inherent characteristics of the infecting HSV strain contribute to disease outcome following neonatal infection.

**Keywords.** herpes simplex virus; encephalitis; neonate; neurovirulence.

Each year approximately 14,000 infants are infected with herpes simplex virus (HSV) in the perinatal period [1], typically due to maternal genital infection and shedding of HSV-1 or HSV-2 at the time of birth. Infected infants follow different clinical courses [2, 3]. About half will experience only noninvasive disease affecting the skin, eyes, and mouth (SEM disease). However, 30% will experience invasive infection of the central nervous system (CNS disease), with another 15% experiencing invasive systemic infection (disseminated disease) with CNS involvement. While treatment of all HSV-infected neonates with acyclovir has dramatically decreased deaths [4], survivors of invasive CNS disease often experience permanent neurologic deficits [5–7]. The personal and societal costs of this lost developmental potential, over the lifetime of these individuals, cannot be overstated.

The factors that promote HSV infection of the neonatal brain are not well understood. Previous studies have investigated the causes of HSV encephalitis in adults and older children, and have shown that individuals with rare host defects in specific innate immune pathways may be predisposed to CNS infection [8, 9]. However, the vast majority of HSV encephalitis in adults and older children is not explained by a known host immune defect [10], and no such host defect has been shown to contribute to HSV encephalitis in neonates. Rather, the epidemiology of neonatal disease, with more than 45% of HSV-infected neonates experiencing CNS involvement, makes it unlikely that host genetics alone could account for invasive forms of infection.

Viral variants that impact virulence and disease outcomes have been identified for several pathogens including influenza [11], human immunodeficiency virus (HIV) [12], and others. These viral genotype-to-disease-phenotype comparisons have classically been addressed in small RNA genomes with high levels of variability, while the large DNA genomes of herpesviruses have been considered less diverse. However, the recent work of our laboratory and others has shown that herpesvirus genetic variation exists between hosts, as well as within a given host [13–19]. These data provide rationale for assessing the contribution of HSV genetic variation to clinical manifestations and disease severity in neonatal infection.

We previously examined HSV-2 isolates from a cohort of neonates with diverse disease manifestations [13]. This analysis provided the first understanding of viral genetic variation in the neonatal population. It also identified specific variations associated with CNS disease, suggesting that viral variation may...
contribute to neurologic outcome. However, this study did not address whether CNS disease-associated HSV-2 isolates exhibit increased neurovirulence in support of this hypothesis. Here, we directly assessed the capacity of neonatal HSV-2 isolates to cause neurologic infection in a series of in vitro and in vivo models. We show that clinical HSV-2 isolates, independent of human host variation, are sufficient to recapitulate the relative neurovirulence observed in the human neonatal patient. These findings suggest that inherent characteristics of the infecting HSV-2 strain contribute to disease outcome following neonatal infection.

METHODS

Viruses

Clinical HSV-2 isolates were collected from neonates enrolled in clinical studies [4, 6, 7] by the Collaborative Antiviral Study Group at the University of Alabama at Birmingham and have been previously sequenced and described [13]. Samples were collected from either the cerebrospinal fluid (CSF) or skin at the time of diagnosis. The clinical morbidity score was determined at 12 months of life as previously defined [5, 20]. Viral stocks were prepared by passaging 3 times on MRC-5 cells, and titering on U2OS cells, as previously described [13]. Viral stocks used for intranasal infections received an additional passage on Vero cells, followed by titering on Vero cells, due to slight variation in standard practice between laboratories.

Cell Culture and Neuronal Differentiation

U2OS (American Type Culture Collection [ATCC], HTB-96), Vero (ATCC, CCL-81), and Lund human mesencephalic (LUHMES; ATCC, CRL-2927) cells were cultured under standard conditions. Cell lines were authenticated by ATCC prior to purchase and were confirmed to be mycoplasma free throughout the experiments by periodic testing (LookOut Mycoplasma; Sigma). LUHMES were differentiated as previously described [21, 22]. Briefly, cells were changed to DMEM-F12 media (12634010; Gibco) containing N-2 supplement (17502-048; Invitrogen), GlutaMAX (35050061; Gibco), doxycycline at a final concentration of 1 μg/mL, dibutyryl cyclic AMP at a final concentration of 1 mM (D0627; Sigma), and glial cell-derived neurotrophic factor at a final concentration of 2 ng/mL (212-GD-010; R&D Systems). All experiments were conducted with LUHMES following 5 days of differentiation, at which point they appeared morphologically neuronal with extensive interconnected neural processes, and stained positive for the mature neuronal markers MAP2 and SMI31.

Multistep Growth Curves

HSV-2 growth curves were performed in differentiated LUHMES at multiplicity of infection (MOI) = 0.1. At 1 hour postinfection (hpi) virus was removed and fresh media containing 1% human serum was added to reduce cell-free spread of virus. Virus was collected at each time point and quantified by titering on U2OS cells.

Immunocytochemistry

Immunocytochemistry analysis was performed as previously described [13]. Infection was detected with anti-HSV primary antibodies (B0114; Agilent Dako). Neuronal processes were detected with a cocktail of anti-MAP2 (Ab5392; Abcam) and anti-SMI31 (801602; Covance) primary antibodies. Images were collected with a Leica DM6000 wide-field microscope and processed with Leica Application Suite software. Exposure, gain, and image processing (ImageJ) was applied identically to all images.

Murine Viral Inoculations

C57BL/6J mice were purchased from Jackson Laboratories and bred at Northwestern University or Dartmouth College. For adult experiments, male and female mice were used in approximately equal numbers, and inoculated at 6–8 weeks of age. For neonate experiments, sex was not determined due to their young age. Neonatal pups were inoculated at 7 days of age for intracranial infections, and 1–2 days of age for intranasal infections, as is standard for each model. For survival experiments, mice were weighed daily over the experimental period indicated, and sacrificed if they lost 30% of their initial weight or displayed severe neurologic symptoms. Organs were harvested at mortality or conclusion of the experiment. For time course experiments, mice were sacrificed and organs harvested at predetermined time points. Each organ was weighed, homogenized, and sonicated prior to titering. When indicated, euthanasia was performed using CO2 and cervical dislocation.

Intracranial Infection

Intracranial infections were performed as previously described [23–25]. After animals were anesthetized, a needle was placed through the right parietal bone, lateral to the sagittal suture and equidistant to the lambda and bregma. A 1000 plaque forming units (PFU) dose was delivered in 10 μL.

Intraperitoneal Infection

Intraperitoneal infections were performed as previously described [26]. A 1000 PFU dose was delivered intraperitoneally in 50 μL.

Intranasal Infection

Intranasal infections were performed as previously described [27]. A 500 PFU (survival) or 1000 PFU (organ titer) dose was delivered intranasally.
Statistics
All statistics were performed using GraphPad Prism 9.0 software. For multiple step growth curves, 2-way ANOVA was performed followed by Sidak’s multiple comparisons test. For survival experiments, log-rank analysis was performed. For organ titer comparison following death or conclusion of the experiment (bimodal data), organs were categorized as containing virions above the limit of detection or not, and 1-way Fisher exact test was performed. For organ titer comparison prior to death, multiple unpaired t tests with Welch correction were performed.

Study Approval
The protocols for animal use were approved by the Institutional Animal Care and Use Committee of Northwestern University (No. IS00016935) or Dartmouth College (No. 00002151). All animal experiments were carried out in adherence to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

RESULTS
CNS-Derived HSV-2 Exhibits Enhanced Spread in Human Neuronal Cultures
To determine whether HSV-2 isolated from neonates with neurologic versus nonneurologic clinical outcomes demonstrated enhanced spread in human neuronal cultures, we focused on isolates at opposite ends of the clinical spectrum. CNS11 was isolated directly from the CSF of a neonate with severe encephalitis and poor neurologic outcome, while SEM02 was isolated from the skin of a neonate with skin-limited disease and no long-term impairment (Table 1) [13]. Each was used to infect differentiated LUHMES cells (Figure 1A) [21, 22]. Cells were infected at a low MOI (MOI = 0.1) to assess differences over multiple rounds of viral replication and spread through the neuronal network (Figure 1B and 1C). At 72 hpi the extent of neuron-to-neuron spread was measured either quantitatively by titer (Figure 1B) or qualitatively by immunofluorescence (Figure 1C). Infectious virions recovered were similar at 2, 6, and 12 hpi, confirming that equivalent amounts of each virus were present following the initial infection, and after a single cycle of replication (Figure 1B). However, by 72 hpi, after multiple rounds of replication and spread, CNS11 achieved statistically higher viral loads than SEM02. Next, infected LUHMES were fixed at 24, 48, and 72 hpi and subjected to immunocytochemistry analysis using antibodies directed against total HSV (Figure 1C). By 48 hpi a greater number of infected cells arose from each single infectious focus of CNS11 as compared to SEM02. By 72 hpi CNS11 consumed each culture and often resulted in the breakdown of neuronal processes. We also performed similar infections in a second human neuronal model. When differentiated SH-SY5Y neuroblastoma cells (Supplementary Figure 1A) [28] were infected at a low MOI, CNS11 similarly produced a statistically higher titer at 72 hpi than SEM02 (Supplementary Figure 1B). Taken together, these data showed that both viruses were competent to enter neurons, replicate with equal kinetics during early infection, and achieve a productive, spreading infection within neuronal cultures. However, the neurologic disease-associated isolate CNS11 exhibited enhanced spread as compared to the skin-limited SEM02, indicating a potential advantage in a neurologic environment.

CNS-Derived HSV-2 Promotes Neurologic Disease Following Murine Peripheral Inoculation
These in vitro data suggested CNS11 may promote neurologic infection via an increased ability to enter or spread within the CNS. Therefore, we tested its in vivo neurologic phenotypes following peripheral inoculation. First, intraperitoneal infections were performed in adult mice, which result in presumed hematogenous dissemination of virus [26, 29]. After 2 weeks, only 9% of animals infected with the skin-derived SEM02 had died, as compared to 55% of animals infected with the

Table 1. Clinical and Experimental Characteristics of Each Neonatal HSV-2 Isolate

<table>
<thead>
<tr>
<th>Clinical HSV-2 Isolate</th>
<th>Human Disease(s) at Diagnosis</th>
<th>Human Isolate Source</th>
<th>Human Morbidity Score</th>
<th>Mouse IC Model No. Died/ Total (%)</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEM18</td>
<td>SEM</td>
<td>Skin</td>
<td>1/9 (11)</td>
<td>MK106004</td>
<td></td>
</tr>
<tr>
<td>SEM13</td>
<td>SEM</td>
<td>Skin</td>
<td>5/9 (56)</td>
<td>MK106003</td>
<td></td>
</tr>
<tr>
<td>SEM02</td>
<td>SEM</td>
<td>Skin</td>
<td>5/9 (56)</td>
<td>MK106002</td>
<td></td>
</tr>
<tr>
<td>DISS14</td>
<td>DISS + CNS</td>
<td>CSF</td>
<td>2</td>
<td>6/9 (67)</td>
<td>MK106000</td>
</tr>
<tr>
<td>CNS03</td>
<td>CNS</td>
<td>Skin</td>
<td>4</td>
<td>7/9 (78)</td>
<td>MK105995</td>
</tr>
<tr>
<td>DISS29</td>
<td>DISS + CNS</td>
<td>Skin</td>
<td>3</td>
<td>8/9 (89)</td>
<td>MK106001</td>
</tr>
<tr>
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<td>4</td>
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</tr>
<tr>
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<td>Skin</td>
<td>4</td>
<td>9/9 (100)</td>
<td>MK105999</td>
</tr>
<tr>
<td>CNS11</td>
<td>CNS</td>
<td>CSF</td>
<td>4</td>
<td>9/9 (100)</td>
<td>MK105996</td>
</tr>
</tbody>
</table>

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; DISS, disseminated; IC, intracranial; SEM, skin, eyes, and mouth.
CNS-derived CNS11 (Figure 2A). Both male and female mice were similarly affected (Supplementary Figure 2). All animals that died had high viral loads within the brain at the time of death (Figure 2B). However, significantly more animals developed neurologic infection following inoculation with CNS11 as compared to SEM02 (73% vs 9%; Figure 2B), with nearly half of CNS11-infected animals experiencing infection only within the brain following hematogenous dissemination (Figure 2B). Flank infections were also performed in adult mice, which assessed the ability of virus to enter and replicate within the spinal cord to produce hindlimb paralysis [30]. Mice were significantly more likely to experience hindlimb paralysis following CNS11 as compared to SEM02 inoculation (Supplementary Figure 3A). Notably, infection with either virus resulted in indistinguishable inoculation site skin disease prior to neurologic symptom onset (Supplementary Figure 3B). To assess peripheral infection in neonatal mice, intranasal inoculations were performed [27]. Consistent with the pattern of virulence observed following adult peripheral inoculation, 88% of CNS11-infected mice died compared to only 40% of SEM02-infected mice (Figure 3A). Comparable viral loads were detected within the lungs and other peripheral organs of both CNS11- and SEM02-infected mice 3 days following intranasal inoculation (Figure 3B). However, viral loads within the brain were

Figure 1. CNS-derived HSV-2 exhibits enhanced spread in human neuronal cultures. A, Human LUHMES were differentiated for 5 days in the presence of doxycycline to produce neuron-like cells; 10× images of replicating vs terminally differentiated cells are shown. B and C, Differentiated LUHMES were infected at multiplicity of infection = 0.1 and samples were harvested at each time point. B, Virus was quantified by titer. Triplicate data are shown as mean ± SEM; *P = .01. C, In parallel experiments, HSV-positive cells (green) were evaluated by immunofluorescence. Neuronal processes are counterstained with MAP2 and SMI31 (red), and cell nuclei with DAPI (blue); 5× images are shown. Images are representative of results from 3 independent experiments. Abbreviations: CNS, central nervous system; DAPI, 4',6-diamidino-2-phenylindole; hpi, hours postinfection; HSV, herpes simplex virus; LUHMES, Lund human mesencephalic cells; PFU, plaque-forming unit; SEM, skin, eyes, and mouth.
significantly higher following CNS11 as compared to SEM02 inoculation (Figure 3B). Taken together, these data indicate that CNS11 is able to enter or replicate within the CNS following peripheral inoculation more readily than SEM02, and suggest a possible preference for the CNS relative to other organs.

**CNS-Derived HSV-2 Promotes Neurologic Disease Following Murine Intracranial Inoculation**

Evaluation of multiple peripheral models of infection provides clinical relevance, but does not distinguish between a viral advantage in entry verses replication within the CNS. To determine whether CNS11 was more neurovirulent than SEM02 in the absence of peripheral barriers to CNS entry, we assessed these HSV-2 isolates following direct intracranial inoculation of adult mice [23–25]. All mice infected with CNS11 died by 9 days postinfection (dpi) as compared to half of those infected with SEM02 (Figure 4A). Both male and female mice were similarly affected (Supplementary Figure 4A). All animals who died experienced consistent weight loss (Supplementary Figure 4B). Notably, consistently high viral loads were detected within the brains of CNS11-infected animals at the time of death (Figure 4B). By contrast, animals who survived direct intracranial injection of SEM02 were able to clear infectious virus from the brain by the conclusion of the experiment (Figure 4B).
define the kinetics of viral replication and clearance following intracranial infection, we performed inoculations with either CNS11 or SEM02, and harvested brain tissue over time (Figure 4C and 4D). SEM02 viral load lagged behind CNS11 at 3 and 5 dpi (Figure 4C). During this time CNS11-infected animals exhibited increased neurologic symptoms, including repetitive circling and severe agitation (Supplementary Figure 4C). To specifically assess the time course of SEM02 clearance from the brain, we harvested brain tissue over a later time course from animals predicted to survive based on lack of weight loss at 7 dpi. All predicted survivors were able to clear virus between 9 and 12 dpi (Figure 4D).

Next, intracranial infections were performed in 7-day-old mice to determine whether CNS11 and SEM02 also produced different neurologic phenotypes in neonatal animals [24, 25]. Infection with CNS11 resulted in rapid death of all animals by 4 dpi (Figure 5A), and uniformly high viral loads within the brain at the time of death (Figure 5B). In this neonatal model, SEM02 also resulted in the death of all infected animals, but occurred statistically significantly later than in those infected with CNS11 (Figure 5A). Interestingly, several SEM02-infected animals had no detectable virus within the brain at the time of death (Figure 5B), mimicking the clearance of SEM02 seen in the adult model. Animals that cleared infectious virus did not survive longer than those with high viral loads (Supplementary Figure 5). Overall, neonatal brains contained approximately 10-fold more infectious virus at the time of death as compared to adult brains infected with the same amount of input virus (Figures 4B and 5B).

These data showed that CNS11 consistently resulted in neurologic infection and death once peripheral barriers to CNS entry were breached, while SEM02 virions were often cleared from the brain. Notably, viral clearance late in infection led to survival in adult animals, but not in neonatal animals. This suggests a role for virus-independent neonatal mortality, perhaps due to an overwhelming immune response necessary for viral clearance that cannot be tolerated by the neonatal brain.

Multiple HSV-2 Strains Isolated From Neonates With CNS Disease Exhibit Superior Neurovirulence

These experiments evaluating the in vivo phenotype of 2 HSV-2 strains isolated from neonates on opposite ends of the clinical neurologic spectrum suggest that inherent viral differences can contribute to neurovirulence. To determine if the ability of a particular virus to recapitulate clinical disease manifestations in a murine model is more generalizable, we subjected 7 additional HSV-2 isolates collected from neonates with differing severity of disease (Table 1) [13] to intracranial infection of adult mice (Figure 6A and 6B). In addition to CNS11, CNS12, CNS17, and DISS29 caused severe neurologic morbidity in their human source patients (Table 1). All of these HSV-2 isolates caused significant mortality and high CNS viral loads following murine intracranial infection (Figure 6A and 6B). In addition to SEM02, SEM13 and SEM18 were collected from neonates with skin-limited disease (Table 1). These isolates resulted in significantly lower rates of mortality and higher rates of viral clearance from the brain (Figure 6A and 6B). Notably, DISS14 caused disseminated disease with CNS involvement in its human source patient, but only mild neurologic morbidity (Table 1). This HSV-2 isolate resulted in intermediate disease following murine intracranial infection (Figure 6A and 6B).

Neurologic infection following peripheral inoculation was also assessed with additional representative HSV-2 strains (Supplementary Figure 6). Following neonatal intranasal infection, CNS11, CNS12, DISS29, and CNS03 resulted in significantly greater mortality than SEM02 (Supplementary Figure 6A), although not significantly higher brain titers at

![Figure 3](https://academic.oup.com/jid/advance-article-fig/doi:10.1093/infdis/jiac151/6572279) CNS-derived HSV-2 promotes neurologic disease following intranasal inoculation in neonatal mice. Neonatal mice were infected intranasally with 500 PFU HSV-2 (n = 8–10 mice/virus over 2 independent experiments) and (A) monitored daily for survival; **P = .003. B, In parallel experiments, mice were infected with 1000 PFU HSV-2 (n = 5 mice/virus over 2 independent experiments) and organ tissue was collected at 3 DPI for quantification of viral titer. Dashed line represents limit of detection. *P = .04. Abbreviations: CNS, central nervous system; dpi, days postinfection; HSV, herpes simplex virus; ns, not significant; PFU, plaque-forming unit; SEM, skin, eyes, and mouth.
3 dpi (Supplementary Figure 6B). Notably, CNS03 more closely recapitulated the significant neurologic morbidity produced in its human source patient (Table 1) in this model as compared to the adult intracranial infection model. Finally, adult intraperitoneal infection was performed with all clinical isolates, but only CNS11 infection produced significantly greater death or CNS viral loads as compared to other isolates (Supplementary Figure 6C and 6D).

These data showed that virus alone, independent of human host factors, can recapitulate the relative neurovirulence...
observed in a neonatal patient. While the neurologic outcome of neonatal HSV-2 infection is almost certainly multifactorial, these data indicate that viral variation contributes to clinical outcome.

DISCUSSION

Neonatal HSV infection results in widely disparate neurologic outcomes, which are often assumed to be driven by human host variation. We utilized a series of neurologically relevant in vitro and in vivo models to evaluate the neurovirulence of clinical HSV-2 isolates independent of human host variation. We found that CNS disease-associated isolates were significantly more likely to cause neurologic infection and death as compared to skin-limited isolates in multiple murine models of encephalitis, in both adult and neonatal animals, performed in multiple laboratories with separately grown viral stocks. Our results suggest an inherent difference in the potential for a given HSV-2 strain to cause invasive neurologic disease in the human neonate.

This study represents the largest set of HSV isolates obtained from neonates that have been evaluated genotypically [13], and the data presented here provide an important link to neurologic phenotype. We previously identified several viral variations in HSV-2 proteins associated with cell-to-cell spread, which were unique to isolates cultured directly from the CSF (gK, gI, gG, US2, and UL8), and other variations associated with CNS disease (UL24) or disseminated disease (UL20) more broadly. However, this analysis could only assess for genotype-to-human-phenotype links driven by multifactorial clinical outcomes, or sample characteristics of unknown significance such as location of virus collection. The current study provides additional data, allowing for comparative genomic analysis of the most neurovirulent isolates, unbiased by host or clinical characteristics. For example, we can compare the previously described genic regions [13] to identify amino acid variations that occur only in isolates causing significantly greater animal mortality (CNS11, CNS12, CNS17, and DISS29) as compared to SEM isolates (Figure 6A). This analysis reveals 2 new variations of potential importance (Supplementary Figure 7): 1 unique variation occurs in the thymidine kinase (UL23) and another in the capsid protein VP5 (UL19). Both proteins have been previously shown to be important for neurovirulence [31–33]. Ongoing research will use this method to identify additional monogenic associations in intergenic and highly repetitive regions of the viral genome not previously analyzed, and identify polygenic links by extending these analyses to additional neonatal isolates.

In addition to these genomic links, evaluation of viral phenotype in multiple models of encephalitis provides further insights. Notably, all CNS-disease-associated isolates tested
exhibited neurovirulence, regardless of whether the isolate was obtained from the CSF or skin of the patient. This suggests that inherent characteristics of the infecting strain drive neurovirulence, rather than these features being selected for in the viral population only after entry and replication within the human neonatal brain. Our results also identified nuanced differences between neurovirulent strains. While CNS11 exhibited an advantage in establishing CNS infection in all murine models of encephalitis, the neurovirulence of other CNS-disease–associated isolates was dependent on the route of inoculation. These results highlight the importance of peripheral barriers to CNS infection and suggest that multiple viral factors may contribute to the capacity for neurologic disease by different mechanisms, either independently or in combination.

It is important to note that human clinical disease outcome is almost certainly multifactorial, including viral virulence, route of infection, viral dose, gestational age, host immune competence, and presence of maternal antibody, among other factors. Our data indicate that when other factors are controlled for, viral variation alone is sufficient to reproduce the relative neurovirulence observed in the human patient, suggesting it is an important contributor. Notably, while neonates often

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**Figure 6.** Multiple HSV-2 strains isolated from neonates with CNS disease exhibit superior neurovirulence. Adult mice were infected intracranially with 1000 PFU HSV-2 (n = 9 mice/virus) and (A) monitored daily for survival. *P < .02. B, Virus was quantified in brain tissue by titer at the time of death or conclusion of the experiment. Dashed line represents limit of detection. Animals are categorized by presence or absence of detectable PFU in brain; *P < .05. Abbreviations: CNS, central nervous system; DISS, disseminated; dpi, days postinfection; HSV, herpes simplex virus; PFU, plaque-forming unit; SEM, skin, eyes, and mouth.
experience severe neurologic outcomes, their mothers who are presumably genitally infected with the same viral strain do not —although notable rare exceptions involving dual mother-child fatality do exist [14, 34]. It is possible that inherent neurovirulent characteristics are revealed preferentially in neonates because peripheral barriers are more routinely breached. This may occur in the setting of increased blood-brain barrier permeability associated with gestational age, or penetration of epithelial barriers with scalp electrode placement during birth monitoring, both known risk factors for neonatal CNS infection [6, 35]. Our results also showed increased viral load following intracranial infection of neonatal animals as compared to adult animals, as well as death of neonates even following viral clearance. These data may indicate an exaggerated immune response as a contributor to neonatal mortality, and suggest that a complete evaluation of the neuroimmune response elicited by various viruses would provide valuable insights on how age-specific differences contribute to poor neonatal outcomes. Understanding which viral strains have the greatest inherent potential for harm, and ultimately the mechanism by which this occurs, could lead clinicians to provide longer courses of antiviral treatment or suppression in specific circumstances, or target a dysregulated neuroimmune response to provide therapeutic benefit [36]. Our unique approach utilizes natural forward genetics, clinical observations, and animal modeling to elucidate the key virus-host interactions in the neonatal brain that determine clinical outcome.

Supplementary Data
Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes
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