HSV targeting of the host phosphatase PP1α is required for disseminated disease in the neonate and contributes to pathogenesis in the brain

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Edited by Bernard Roizman, The University of Chicago, Chicago, IL, and approved November 10, 2015 (received for review July 2, 2015)

Newborns are significantly more susceptible to severe disease after infection with herpes simplex virus (HSV) compared with adults, with differences in the host response implicated as a major factor. To understand host response differences between these age groups, we investigated the shutoff of protein synthesis by the host and the retargeting of host phosphatase PP1α by the HSV-1 protein γ34.5 for reversal of translational arrest. In a murine newborn model of viral dissemination, infection with the HSV-1 mutant for PP1α binding resulted in complete absence of disease. PP1α-binding mutant HSV-1 replicated in visceral organs early after inoculation, demonstrating that HSV-1 replication requires PP1α-targeting only later in infection. Newborn mice deficient in type I IFN signaling partially rescued the virulence of the PP1α-binding mutant virus, suggesting an IFN-independent role for eIF2α kinases during infection. When we investigated the contribution of PP1α targeting to pathogenesis in the brain, we found that the inability of HSV-1 to bind PP1α increased survival time in both newborn and adult mice. Unlike disseminated disease, type I IFN signaling in the brain was required to attenuate disease following PP1α-mutant virus infection. Furthermore, pharmacologic inhibition of eIF2α dephosphorylation reduced HSV-1 replication in a brain slice culture model of encephalitis. Our findings reveal age-dependent differences in γ34.5 function and tissue-specific reliance on the type I IFN response for protection from HSV disease. These results define an important role for γ34.5 in neonatal infections in contrast to other studies indicating that the autophagy-inhibiting function of γ34.5 is dispensable for pathogenesis in the newborn brain.

HSV-1 | translational arrest | disseminated disease | interferon response

Herpes simplex virus (HSV) infections cause a wide spectrum of outcomes, ranging from asymptomatic acquisition to lethal dissemination and central nervous system (CNS) disease (1). Newborns are particularly susceptible to HSV disease with over 50% of infected newborns progressing to disseminated disease or encephalitis. Of those who survive the initial encephalitis, 2/3 of newborns will go on to have long-term neurologic morbidity (2). This is in contrast to the adult population, where occurrence of HSV encephalitis and disseminated disease are rare and infection with HSV is often subclinical (3). Either serotype of HSV (HSV-1 or HSV-2) may cause disease in newborns, but recent data suggest a rising incidence of HSV-1 genital infection, paralleled by a predominance of HSV-1 as a cause of newborn disease (4, 5). The striking difference in outcomes between adults and neonates infected with HSV suggests an age-dependent difference in susceptibility to disease based on host factors.

It is likely that differences in the immune response in the neonate compared with the adult result in the disparate outcomes following HSV infection, and this was investigated in our current studies.

One of the earliest responses to infection is the type I IFN response and the innate pathways modulated by the IFN-inducible double-stranded RNA-dependent protein kinase R (PKR) system. HSV-1 counters the PKR response in part through production of the viral protein γ34.5 for successful replication in cell culture (6, 7). Mutant viruses deleted for the entire γ34.5 gene are significantly attenuated in vivo, and virulence of γ34.5-null HSV-1 is restored in adult $PKR^{-/-}$ mice in models of ocular and CNS disease (8). Within γ34.5 are domains that specifically target host cell-mediated translational arrest (7, 9), type I IFN response induction through TANK-binding kinase 1 (TBK1) (10), and initiation of autophagy through beclin 1 binding (11, 12). The different functions of HSV-1 γ34.5 have been shown to contribute to replication in cell culture and in some models of HSV disease in the adult; however, evidence suggests that γ34.5 may interact with the host differently in the newborn. We recently demonstrated that the autophagy-inhibiting function of γ34.5 through beclin 1 binding, although important for disease in the adult murine CNS, is completely dispensable for pathogenesis in the neonatal murine brain (13). However, deletion of the entire γ34.5 gene still results in attenuation of HSV in the newborn brain (13), suggesting that there are other functions of γ34.5 that are important for pathogenesis in the neonate.

The shutoff of protein synthesis resulting from eIF2α phosphorylation is an important host response to viral replication and ER stress during infection. There are four serine–threonine eIF2α kinases in mammalian cells (PKR, GCN2, HRI, PERK), two of which have been established to control eIF2α phosphorylation during HSV-1 infection, namely PKR and the endoplasmic reticulum (ER) resident kinase PERK (14). In cells infected with a γ34.5-mutant HSV-1, activation of PKR and subsequent eIF2α phosphorylation is responsible for the cessation of protein synthesis. However, wild-type HSV-1 has evolved an effective strategy through γ34.5 to reverse the translational arrest following eIF2α phosphorylation for successful viral replication. The carboxyl terminus of HSV-1 γ34.5 is homologous to

Significance

The increased severity of herpes simplex virus (HSV) disease in the newborn compared with the adult is a result of complex interactions between the virus and the host response. We studied viral targeting of host-mediated shutoff of protein synthesis during HSV disease in a murine model and found that HSV-1 targeting of the host phosphatase PP1α was required for disseminated disease in the newborn and contributed to HSV encephalitis. Additionally, this report demonstrates that the host response resulting in translational arrest is tissue-specific and also reveals a tissue-specific reliance on the type I IFN response for control of HSV-1. These results provide important insight into the mechanisms of severe HSV disease in the newborn compared with the adult.

Author contributions: D.R.W., W.J.M., and R.L. designed research; D.R.W. performed research; D.R.W., W.J.M., and R.L. analyzed data; and D.R.W., W.J.M., and R.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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the host protein GADD34 (15) and serves as an adaptor molecule to bind both the host phosphatase PP1α and eIF2α, thus targeting eIF2α for dephosphorylation and reversing translational arrest (7, 9, 16, 17). Evidence suggests that PP1α binding by HSV-1 γ34.5 contributes to pathogenesis in an ocular model of HSV disease (18); however, the role of PP1α targeting by HSV in CNS disease or in the unique immune environment of the developing newborn remain unknown.

We demonstrate that the PP1α-targeting function of HSV-1 γ34.5 is required for pathogenesis in a model of neonatal disseminated disease. Investigation of early viral replication in visceral organs demonstrate that PP1α binding by HSV-1 is not required for early replication on the first day after infection, but is required for further replication after day 1 postinfection. Ablation of the type I IFN response only partially restored the virulence of a PP1α-binding–deficient virus, suggesting an IFN-independent contribution of PP1α targeting by HSV-1 to disease in the periphery. When inoculated directly intracranially, the PP1α-binding mutant virus was attenuated in both the neonatal and adult brain, suggesting that translational arrest is important for protection from HSV disease in both age groups. Unlike in the periphery, we show that the attenuation of a PP1α-binding mutant virus is dependent on the type I IFN response in the brain. Additionally, inhibiting inducible eIF2α dephosphorylation during HSV infection of organotypic brain slice cultures reduced viral replication in both age groups. Our findings suggest that viral targeting of PP1α mediated by HSV-1 γ34.5 significantly contributes to disease in both the neonate and the adult and that the host responses that result in translational arrest during infection are tissue-dependent.

Results

HSV Targeting of PP1α Is Required for Disseminated Disease in the Neonatal Mouse. The reversal of translational arrest mediated by HSV-1 γ34.5 binding the host phosphatase PP1α and the eukaryotic initiation factor eIF2α is important for viral replication in cell culture (7, 9) and in a model of HSV keratitis (18). We used a virus that carries specific mutations in γ34.5 that abolish PP1α binding (19), or its marker rescue virus, to test whether PP1α binding by HSV-1 γ34.5 contributes to disseminated disease in the newborn. We inoculated 7-d-old wild-type (WT) mice intraperitoneally (i.p.) with 10^4 plaque forming units (pfu) of either the virus mutant for PP1α binding (ΔPP1α) or its marker rescue (ΔPP1α-R). Interestingly, the ΔPP1α virus was completely attenuated for mortality in the WT neonate with 100% survival in neonatal mice infected with the mutant virus (Fig. 1*A*). As was expected for a WT virus, there was 0% survival in neonatal mice infected with the rescue virus and a median survival time of 5.5 d. At mortality, robust replication of the rescue virus was detected in the visceral organs of mice, including the lung, liver, and spleen, and also in the brain (Fig. 1*B*). At the end of the experimental time course on day 14, there was no detection of viral replication in either the brain or the viscera of neonatal mice infected with the mutant virus ΔPP1α.

The absence of disease in neonatal mice infected with ΔPP1α raised the question of whether there was early replication of ΔPP1α that was rapidly controlled by the host response or if the mutant virus was replication-deficient. To determine the replication dynamics of ΔPP1α compared with its rescue virus in vivo, we assayed for viral replication at days 1, 2, and 3 after i.p. infection. We found that the PP1α-binding mutant virus initially replicated to WT levels on day 1, but had reduced replication

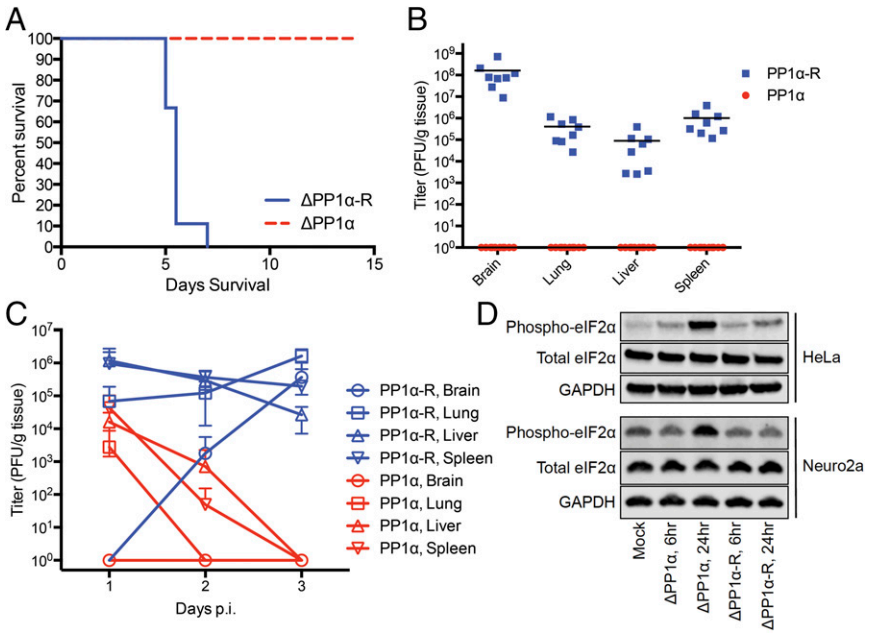

Fig. 1. HSV targeting of the host phosphatase PP1α is required for disseminated disease in the neonatal mouse following i.p. inoculation. (*A*) Survival of 7-d-old neonatal WT mice (n = 9–10 for each group) inoculated i.p. with 10^4 pfu of either a virus mutant in the two amino acids responsible for PP1α binding (ΔPP1α) or its marker rescue virus (ΔPP1α-R). Results shown represent survival data from eight independent inoculations. There was 0% mortality in the neonates infected with ΔPP1α, compared with 100% mortality and a mean survival of 5.5 d in mice inoculated with ΔPP1α-R ($P < 0.0001$). (*B*) Titer of ΔPP1α or ΔPP1α-R in the brain and visceral organs (lung, liver, and spleen) at time of mortality or postinoculation day 14. ΔPP1α was not detected at day 14 after infection in any of the organs assayed, compared with high viral titers in the organs of ΔPP1α-R infected neonates at death (mean titers: brain = 1.6E8 pfu/g; lung = 4.1E5 pfu/g; liver = 8.9E4 pfu/g; spleen = 1.02E6 pfu/g). (*C*) Titer of ΔPP1α or ΔPP1α-R in the brain and visceral organs at days 1, 2, and 3 postinoculation (n = 5 per virus, per time point). There was no statistically significant difference between ΔPP1α and ΔPP1α-R titer in the lung, liver, or spleen on day 1 postinoculation ($P > 0.12$ for all groups). (*D*) Immunoblot of HeLa cells or murine Neuro2a cells infected with either ΔPP1α or ΔPP1α-R at an MOI of 5 and blotted for total eIF2α and phospho-eIF2α (Ser51) at 6 or 24 h postinfection. GAPDH was used as a loading control.

compared with rescue virus on day 2 and no replication detectable in any organs by day 3 (Fig. 1*C*). The rescue virus was detected in all of the visceral organs assayed on day 1 and in the brain by day 2 with increasing replication in the CNS until mortality. The ΔPP1α mutant virus was controlled by the host response in the periphery before it entered the brain and was never detected in the CNS. These data show that infectious virus production on day 1 is not dependent on the PP1α-binding function of γ34.5, but further replication of the virus after day 1 requires PP1α binding. This could reflect a delay in initiating the host responses that result in the control of ΔPP1α virus replication or an insensitivity of the initially infected cells to detecting viral replication and early spread to other cells.

To confirm that the ΔPP1α mutant virus is unable to target eIF2α for dephosphorylation in the context of infection, we determined eIF2α phosphorylation status during infection in vitro in both human and murine cells. Immunoblots of cells infected in vitro with either ΔPP1α or ΔPP1α-R show no difference in eIF2α phosphorylation early in infection at 6 h (Fig. 1*D*). However, at 24 h postinfection, robust phosphorylation of eIF2α in ΔPP1α-infected cells compared with rescue virus-infected cells was detected. PP1α binding by HSV-1 did not result in a global change in the phosphorylation of all PP1α substrates because no difference was detected in the phosphorylation of other PP1α targets including beclin 1, TBK-1, and TAK-1. It has been previously demonstrated that the shutoff of protein synthesis resulting from eIF2α phosphorylation in cells infected with a γ34.5-deficient HSV-1 is associated with the onset of viral DNA synthesis. Our observation that infection with a specific PP1α-binding mutant virus results in eIF2α phosphorylation late in infection is consistent with observations in γ34.5-mutant viruses previously reported by others (20).

Virulence of the PP1α-Binding Mutant Virus Is Partially Restored in Type I IFN Signaling-Deficient Neonatal Mice. The eIF2α kinases, including PKR, are constitutively expressed at low levels in most mammalian tissues. However, PKR expression is strongly induced upon type I IFN stimulation (21) and is subsequently activated in the presence of its activator ligand, double-stranded RNA or PACT, to prevent the translation of viral mRNAs through eIF2α phosphorylation (22, 23). To determine the contribution of the type I IFN response to the attenuation of the PP1α-binding mutant virus during disseminated disease, we inoculated type I IFN receptor knockout (IFNAR KO) neonatal mice intraperitoneally with either the ΔPP1α mutant virus or its rescue, ΔPP1α-R. Both ΔPP1α and ΔPP1α-R–infected IFNAR KO neonatal mice exhibited 100% mortality; however, neonatal mice infected with the PP1α-binding mutant virus had a significantly longer survival time of 5.25 d, compared with 3 d in the ΔPP1α-R–infected group (Fig. 2*A*). Furthermore, despite succumbing to infection days earlier than the neonates infected with ΔPP1α, those infected with the rescue virus had significantly higher viral replication in the lung, liver, and spleen at morality (Fig. 2*B*). These data suggest that, although the type I IFN response significantly contributes to the attenuation of a PP1α-binding mutant HSV-1, there is an IFN-independent role for PP1α targeting by HSV-1 in disseminated disease. This is consistent with IFN-independent eIF2α kinase activity through PKR and PERK. Although PKR expression is greatly induced through IFN signaling, it does not require IFNα/β-receptor ligand binding and signaling through JAK/STAT to be activated (24), and this could explain in part the attenuation of the ΔPP1α mutant virus in IFN-signaling–deficient neonatal mice.

HSV Targeting of PP1α Contributes to Mortality and Viral Replication in the CNS of Both Neonatal and Adult Mice. In a model of neonatal disseminated HSV disease, replication of a mutant virus unable to target the host phosphatase PP1α was controlled early in infection before the virus entered the central nervous system (Fig. 1). In previous studies, we have shown that, unlike HSV disease in the adult brain (11), inhibition of autophagy through beclin 1 binding by γ34.5 was completely dispensable for pathogenesis in the neonatal brain (13). We sought to determine whether the PP1α-binding function of γ34.5 contributed to CNS disease in the neonate. Neonatal and adult mice were inoculated directly intracranially (i.c.) with either the ΔPP1α mutant virus or its rescue. Similar to what is expected from infection with WT HSV-1, there was 100% mortality in neonatal mice infected with ΔPP1α-R (Fig. 3*A*). Interestingly, neonatal mice inoculated with the ΔPP1α mutant virus had a significantly delayed time to mortality with a mean survival time of 8 d, compared with 4.5 d in the neonates infected with the rescue virus. The ΔPP1α virus carrying a specific mutation in the PP1α-binding region of γ34.5 exhibited a similar survival curve to knockout of the entire γ34.5 gene (13), with infection by both mutants resulting in 100% mortality. Despite a significant gain in survival time, replication at mortality was similar between the ΔPP1α mutant virus and ΔPP1α-R (Fig. 3*B*). Infection of the adult brain yielded similar results with a longer mean survival time and greater overall survival in mice infected with ΔPP1α compared with ΔPP1α-R (Fig. 3*C*). These data suggest that, unlike inhibition of autophagy, viral targeting of PP1α contributes to CNS pathogenesis in both age groups.

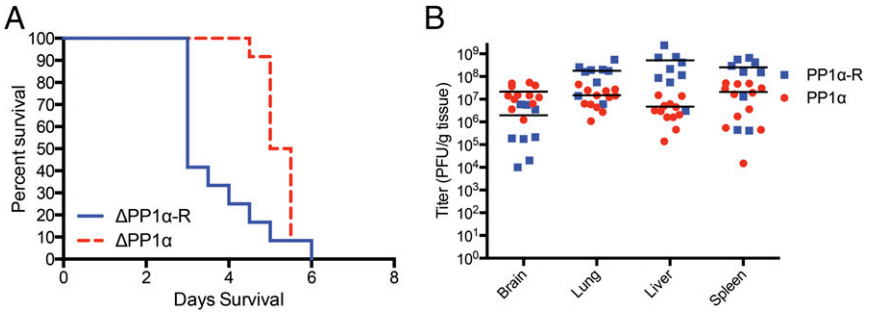

Fig. 2. Virulence of the PP1α-binding mutant virus is partially restored in neonates lacking intact type I IFN signaling following i.p. inoculation. (*A*) Survival of 7-d-old IFNAR KO littermates ($n = 11$ for each group) after i.p. inoculation with 10^4 pfu of either the ΔPP1α mutant virus or its rescue, ΔPP1α-R. Results shown represent survival data combined from 10 different inoculations. Log-rank analysis showed a statistically significant delay in time to mortality for IFNAR KO newborns inoculated with ΔPP1α compared with ΔPP1α-R (median survival: ΔPP1α-*R* = 3 d; ΔPP1α = 5.25 d; $P < 0.01$). (*B*) Titer of ΔPP1α or ΔPP1α-R in the brain and visceral organs (lung, liver, and spleen) at time of mortality in IFNAR KO mice infected i.p. The ΔPP1α-R virus replicated to higher titers than the ΔPP1α mutant in the lung, liver, and spleen (lung: $P = 0.003$; liver: $P = 0.03$; spleen: $P = 0.004$). Replication in the brain at mortality was higher in the ΔPP1α than the ΔPP1α-R group ($P = 0.009$).

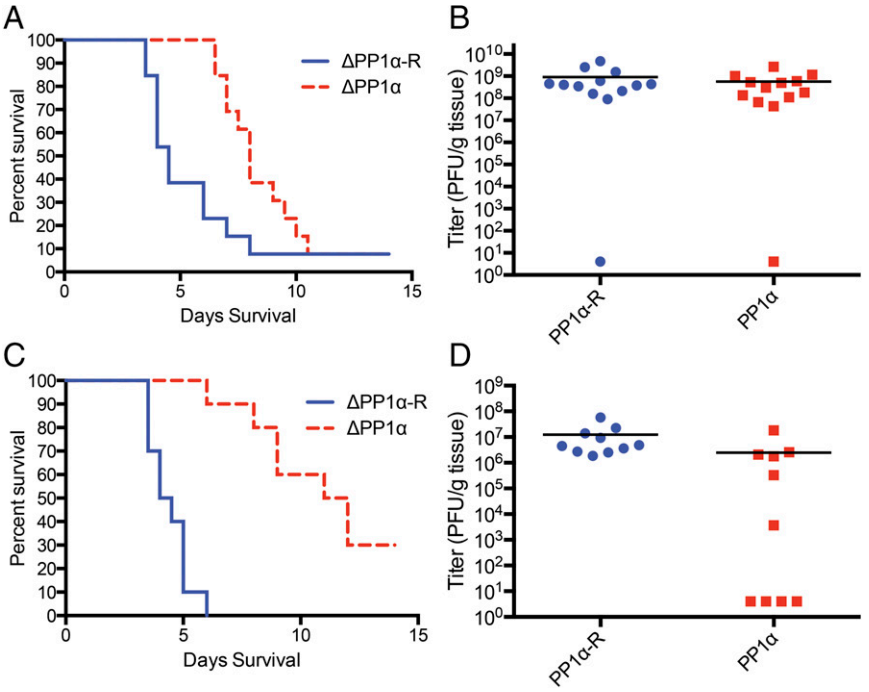

Fig. 3. HSV-1 targeting of PP1α contributes to pathogenesis in the CNS of both neonatal and adult mice following i.c. inoculation. (*A*) Survival of 7-d-old WT littermates inoculated i.c. with 10^2 pfu of either the PP1α-binding mutant (ΔPP1α: $n = 13$) or its rescue virus (ΔPP1α-R: $n = 13$). Results shown represent survival data combined from eight independent inoculations. Log-rank analysis showed a statistically significant delay in time to mortality for WT newborns inoculated with ΔPP1α compared with ΔPP1α-R (median survival: ΔPP1α-*R* = 4.5 d and ΔPP1α = 8 d; $P = 0.01$). (*B*) Titer of ΔPP1α or ΔPP1α-R in the brains of neonatal mice at mortality. There was no statistically significant difference in replication between the two viruses at mortality (mean titers: ΔPP1α-*R* = 5.2E7 pfu/g and ΔPP1α = 5.9E7 pfu/g; $P = 0.79$). (*C*) Survival of adult WT mice inoculated i.c. with 10^4 pfu of either ΔPP1α or ΔPP1α-R ($n = 10$ each group). Results shown represent survival data from eight independent inoculations. Log-rank analysis showed a statistically significant delay in time to mortality after inoculation with ΔPP1α (median survival = 11.5 d) compared with ΔPP1α-R ($P < 0.0001$; median survival = 4.25 d). (*D*) Titer of ΔPP1α or ΔPP1α-R in the brains of WT adult mice at mortality. There was no statistically significant difference in replication between the two viruses at mortality (mean titers: ΔPP1α-*R* = 1.2E7 pfu/g and ΔPP1α = 2.5E6 pfu/g; $P = 0.10$).

Full Virulence of the PP1α-Binding Mutant HSV Is Restored in the CNS of Type I IFN Signaling-Deficient Neonatal and Adult Mice. Our previous studies demonstrated that, unlike the adult, the type I IFN response does not contribute to the control of WT HSV-1 in the newborn brain (13). We inoculated IFNAR KO neonatal and adult mice intracranially to determine if the attenuation of the ΔPP1α mutant virus in the brain was dependent on the type I IFN response for the two age groups. Unlike what was observed after peripheral inoculation (Fig. 2), there was complete restoration of the virulence of the ΔPP1α mutant to WT levels in both IFN signaling-deficient neonatal (Fig. 4*A*) and adult (Fig. 4*C*) mice. Furthermore, viral replication in the brain was the same for ΔPP1α and ΔPP1α-R in both age groups (Fig. 4 *B* and *D*). This result is also consistent with similar replication of ΔPP1α and ΔPP1α-R in the brains of IFNAR KO neonates after i.p. inoculation (Fig. 2*B*). These data suggest that, although the type I IFN response does not contribute to the control of WT HSV-1 in the neonatal brain, the IFN response is not completely absent because it does contribute to the attenuation of the ΔPP1α virus in the neonatal brain. Additionally, our data suggest that, although there is an IFN-independent role for viral targeting of PP1α in the periphery, signaling through type I IFN was required to attenuate disease following PP1α-mutant virus infection in the brain.

Our data suggest a tissue-dependent contribution of the type I IFN response to control of a PP1α-binding mutant virus and tissue-specific differences in the host responses that result in the cessation of protein synthesis. To determine whether the basal expression levels of the regulators of eIF2α phosphorylation may explain, in part, our observations across different tissue types in the neonate, we performed immunoblots for PKR and the PP1α regulatory subunits CreP (PPP1R15B) and GADD34 in the brain, lung, liver, and spleen (Fig. 4*E*). Although basal expression of CreP and GADD34 was similar across the brain and peripheral organs, expression of PKR was significantly higher in the lungs, a site of early HSV-1 replication in the periphery (Fig. 1*C*), compared with the brain. Similar basal expression of PKR across the brain, liver, and spleen suggests a role for other regulators of eIF2α phosphorylation, and possibly tissue-specific differences in the induction of PKR, to explain the differential attenuation of the PP1α-binding mutant virus in the brain compared with the periphery. These results suggest that, at least in the lung, some cells initially infected with HSV-1 express higher levels of PKR compared with the brain (Fig. 4*E*), and this could explain, in part, the significant attenuation of the ΔPP1α virus in some visceral organs compared with the brain in the neonate. One possible explanation is that the low basal expression of the other eIF2α kinases and differential induction of those kinases during infection in the neonatal brain may be insufficient to produce enough phosphorylated eIF2α to shut off translation as effectively as the peripheral organs.

Inhibiting eIF2α Dephosphorylation Decreases Viral Replication in Vitro and in an ex Vivo Organotypic Brain Slice Culture Model of HSV Encephalitis. We have presented evidence that HSV-1 targeting of the host phosphatase PP1α contributes to disease in both the newborn and the adult brain by using a virus mutant for PP1α binding (Fig. 3). The viral protein γ34.5 forms a complex with PP1α and the translation initiation factor eIF2α in vitro (9). Phosphorylated eIF2α is a marker for the shutoff of protein

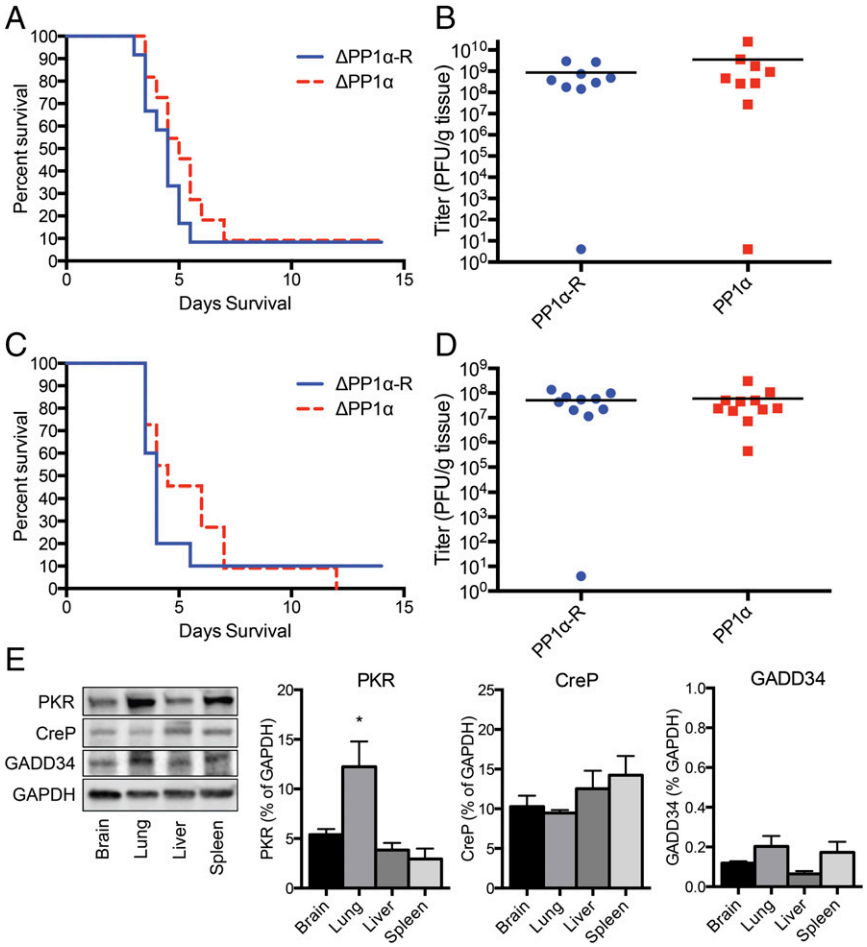

Fig. 4. Full virulence of the PP1α-binding mutant HSV-1 following i.c. inoculation is restored in the CNS of type I IFN-signaling–deficient neonatal and adult mice. (*A*) Survival of 7-d-old IFNAR KO littermates inoculated i.c. with 10^2 pfu of either the ΔPP1α mutant virus ($n = 11$) or its rescue virus ΔPP1α-R ($n = 12$). Results shown represent survival data combined from eight independent inoculations. Log-rank analysis showed no statistically significant difference in time to mortality for IFNAR KO neonates inoculated with either ΔPP1α (median survival = 5 d) or ΔPP1α-R ($P = 0.30$; median survival = 4.5 d). (*B*) Titer of ΔPP1α or ΔPP1α-R in the brains of IFNAR KO neonatal mice at mortality. There was no statistically significant difference in replication between the two viruses at mortality (mean titers: ΔPP1α-*R* = 8.7E8 pfu/g and ΔPP1α = 3.6E9 pfu/g; $P = 0.33$). (*C*) Survival of adult IFNAR KO mice inoculated i.c. with 10^4 pfu of either ΔPP1α ($n = 11$) or ΔPP1α-R ($n = 10$). Results shown represent survival data from 12 independent inoculations. Log-rank analysis showed no statistically significant difference in time to mortality after inoculation with ΔPP1α (median survival = 4.5 d) compared with ΔPP1α-R ($P = 0.50$; median survival = 4.0 d). (*D*) Titer of ΔPP1α or ΔPP1α-R in the brains of IFNAR KO adult mice at mortality. There was no statistically significant difference in replication between the two viruses at mortality (mean titers: ΔPP1α-*R* = 5.2E7 pfu/g and ΔPP1α = 6.0E7 pfu/g; $P = 0.79$). (*E*) Representative immunoblot (*Left*) and densitometry (*Right*) (mean values ± SEM) of whole-organ homogenates from uninfected 7-d-old neonatal and 10-wk-old adult mice ($n = 4$ in each group). Expression of PKR was significantly higher in the lung compared with other organs (lung vs. brain: $P = 0.02$; lung vs. liver: $P = 0.007$; lung vs. spleen: $P = 0.004$; all other comparisons: $P > 0.05$). Expression of CreP and GADD34 was not significantly different across the brain, lung, liver, and spleen (all comparisons: $P > 0.05$).

synthesis in the host cell, and, taken together, this translational arrest suggests that the phosphorylation status of eIF2α contributes to the successful replication of HSV-1. To test the contribution of eIF2α phosphorylation to viral replication, we infected HeLa cells with the rescue virus and treated with either salubrinal, a selective inhibitor of eIF2α dephosphorylation (25), or vehicle control. Treatment with 10 μM salubrinal reduced viral titer 10-fold compared with vehicle control by 72 h, and treatment with 50 μM salubrinal, previously shown to result in robust phosphorylation of eIF2α while maintaining cell viability in vitro (25), resulted in an approximately 10,000-fold decrease in viral titer by 72 h (Fig. 5*A*). The reduction of viral titer after treatment with salubrinal in vitro suggests a significant contribution of inducible eIF2α phosphatase activity to the replication of HSV-1 and is consistent with observations reported by others (26).

Organotypic brain slice cultures have the potential to be a valuable model for studying viral encephalitis because, unlike cell culture systems, they maintain the normal tissue architecture and interactions between neural cells that may contribute to pathogenesis (27, 28). Importantly, organotypic brain slice cultures have previously been used to investigate neurotropism differences between neonate and adults as a model of HSV encephalitis (29). To test the contribution of inducible eIF2α dephosphorylation to viral replication in a model of CNS disease, we infected ex vivo organotypic brain slice cultures from neonatal and adult mice with HSV-1 and treated them with salubrinal or vehicle control. Viral replication in both the neonate and the adult brain slice cultures was significantly reduced with increasing doses of salubrinal compared with vehicle control (Fig. 5 *B* and C). The reduction in viral titer after salubrinal treatment was not due to cell toxicity because there was no discernible difference in cell viability between salubrinal and DMSO control-treated brain slice cultures as determined by the MTT assay. These data suggest that phosphorylation of eIF2α results in reduced viral replication in an ex vivo model of HSV-1 encephalitis. The host phosphatase PP1α forms a complex with

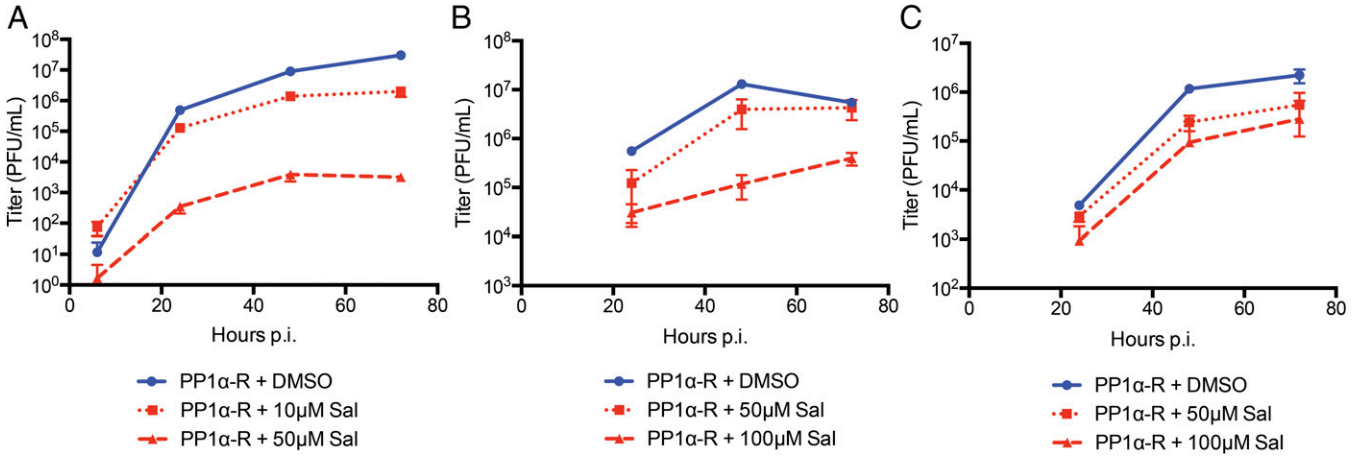

Fig. 5. Inhibition of eIF2α dephosphorylation decreases HSV-1 replication in vitro and in an ex vivo organotypic brain slice culture model of HSV encephalitis. (*A*) HeLa cells treated with 10 or 50 μM salubrinal displayed reduced viral replication compared with DMSO (vehicle-only) treated cells at 24, 48, and 72 h ($P < 0.05$ at all points; $n = 4$ each point). (*B*) Neonatal brain slice cultures infected in triplicate with 10^6 pfu of ΔPP1α-R were treated with DMSO, 50 μM salubrinal, or 100 μM salubrinal. Salubrinal-treated brain slice cultures exhibited decreased viral replication compared with DMSO-treated controls at 24 h ($P = 0.03$ for 50 μM sal; $P = 0.003$ for 100 μM sal), at 48 h ($P = 0.03$ for 50 μM sal; $P < 0.001$ for 100 μM sal), and at 72 h ($P = 0.006$ for 100 μM sal) postinfection. (*C*) Adult brain slice cultures infected in triplicate with 10^6 pfu of ΔPP1α-R were treated with DMSO, 50 μM salubrinal, or 100 μM salubrinal. Salubrinal-treated brain slice cultures exhibited decreased viral replication compared with DMSO-treated controls at 48 h ($P < 0.001$ for both 50 and 100 μM sal) and at 72 h ($P = 0.04$ for 100 μM sal) postinfection.

its nonenzymatic cofactor GADD34 during cell stress or with the constitutively active CreP (26) to mediate dephosphorylation of eIF2α. Salubrinal inhibits dephosphorylation of eIF2α mediated by both phosphatase complexes in vitro (25). The sequence homology between the C terminus of HSV-1 γ34.5 and GADD34 makes it a likely target of salubrinal and probably contributes to the effectiveness of salubrinal in reducing HSV-1 replication in brain slice cultures.

Discussion

We report that HSV-1 targeting of the host phosphatase PP1α by γ34.5 for the reversal of host-mediated translational arrest is required to cause disseminated disease in the neonatal mouse (Fig. 1). We also demonstrate that, although not required to cause disease in the CNS, PP1α targeting by the virus significantly contributes to HSV pathogenesis in the brains of both adult and neonatal mice (Fig. 3). Although the type I IFN response significantly contributed to the control of a virus mutant for PP1α binding, ablation of this response did not entirely restore the virulence of the mutant virus during disseminated disease (Fig. 2), suggesting an IFN-independent role for PP1α targeting by HSV-1 in the visceral organs. In contrast, virulence of a PP1α-binding mutant virus was completely restored to WT levels in the CNS of IFNAR KO mice (Fig. 4), demonstrating that HSV targeting of PP1α is dispensable for CNS pathogenesis in IFN signaling-deficient mice and a possible dependence on the type I IFN response for host shutoff of protein synthesis in the brain. Basal expression of PKR was higher in the lungs of neonatal mice compared with the brain, liver, and spleen (Fig. 4*E*). This could explain, in part, the reduced susceptibility of the lung to the PP1α-mutant virus, but also suggests differential PKR induction during infection across tissues and a possible role for the other eIF2α kinases to explain tissue-dependent differences in susceptibility to the ΔPP1α virus. Taken together, these data suggest that the host response that results in translational arrest is tissue-specific. Finally, in an ex vivo organotypic brain slice culture model of HSV encephalitis, we demonstrate that inhibiting eIF2α dephosphorylation with salubrinal treatment reduces HSV-1 replication in the brain.

The multifunctional HSV-1 protein γ34.5 is important for counteracting several different host antiviral pathways for successful viral replication in the nervous system (30, 31). Until recently, it was not known whether HSV manipulates these responses differently in the unique environment of the developing newborn brain. We have previously shown that, although γ34.5 contributes to CNS disease in both neonatal and adult mice, unlike in the adult, the beclin 1-binding function of γ34.5 is completely dispensable for pathogenesis in the newborn brain (13). It is possible that targeting beclin 1 did not alter disease in the newborn brain due to the unique autophagic environment in the developing brain (32), so HSV must rely on other functions of γ34.5 to promote replication in the newborn. We demonstrate that the PP1α-binding function of γ34.5 significantly contributes to disease in both the adult and the neonatal brain. Interestingly, we also show that γ34.5 targeting of PP1α was more important in nonneuronal tissue because a virus mutated for PP1α binding was completely attenuated for disease in a model of HSV dissemination in the neonate. These results are consistent with likely differences in host responses, including eIF2α and its regulators, across different tissue types (Fig. 4*E*). Furthermore, these data reflect decreased neuroinvasion by the PPα-binding–deficient HSV-1 from the periphery, possibly due to rapid control of the virus and decreased viral loads.

The translational arrest observed in cells infected with HSV-1 γ34.5 mutants reflects global cessation of virus and host protein synthesis before completion of the HSV infectious cycle. This process is mediated by phosphorylation of the initiation factor eIF2α by the kinases PKR and PERK, resulting in an increased affinity of eIF2α for the guanine nucleotide exchange factor eIF-2B and sequestration of the two factors in an inactive complex (33). PKR is an IFN-stimulated gene (ISG) in mammalian cells and, although PKR is constitutively expressed in most tissue types, its expression is rapidly up-regulated in cells treated with IFN (22). The virulence of a γ34.5-null virus is restored to WT levels in $PKR^{-/-}$ mice, suggesting that γ34.5 is central to countering the PKR-dependent pathways in the host (8). Taken together with our results demonstrating only partial restoration of virulence with the ΔPP1α mutant virus in IFN-deficient mice (Fig. 2), our current results are consistent with a significant contribution of IFN-independent eIF2α kinase activity during disseminated HSV disease. Importantly, the neonate is under a unique metabolic state in part due to a period of rapid development and organ growth, but also following the sudden interruption in

nutrient supply immediately after birth, resulting in the early neonatal starvation period (32). Although its role during HSV-1 infection has not yet been described, the eIF2α kinase GCN2 is activated when amino acids are limiting during periods of starvation (34), and therefore GCN2 could contribute to translational arrest in the newborn host during infection. The neonatal brain is also in a period of rapid development, and reduced susceptibility to mRNA translational arrest resulting from eIF2α phosphorylation remains a possibility at this developmental age. The reliance on the type I IFN response for the attenuation of a PP1α-binding–deficient HSV-1 in the brains of both age groups suggests a tissue-specific role for the IFN response, which increases the abundance of ISG-encoded proteins, one of which is PKR.

There is some evidence that the type I IFN response is generally blunted in the newborn compared with the adult (35), and we have previously demonstrated that the type I IFN response does not contribute to the control of WT HSV-1 in the newborn brain (13). Interestingly, here we show that the type I IFN response does contribute to the control of HSV-1 in the visceral organs in a model of disseminated disease, with a shorter survival time and higher viral titer in IFN-deficient neonatal mice compared with WT neonatal mice infected with the rescue virus (Figs. 1 and 2). This suggests not only an age-dependent difference in the type I IFN response, but also a tissue-specific reliance on type I IFN for control of HSV disease. A dampened type I IFN response in the brain may be important for proper development of the newborn brain because multiple lines of evidence suggest that altering the cytokine balance in the brain can have profound developmental consequences (36, 37). Specifically, intracranial inoculation of type I IFNs alone in mice has been shown to result in neurologic deficits later in life (38). Taken together, these results may suggest specific dampening of the IFN response in the newborn brain to avoid a cytokine response that could be detrimental to proper development.

Targeting phosphorylated eIF2α, a marker for translational arrest, with a drug that specifically inhibits eIF2α dephosphorylation effectively reduced viral replication in an ex vivo model of HSV encephalitis (Fig. 5). Salubrinal has previously been shown to prevent the dephosphorylation of eIF2α by inhibiting PP1α phosphatase complexes and thereby promoting translational arrest, both during viral replication and the unfolded protein response (UPR) during ER stress in cell culture (25, 26). Given the strong sequence homology of HSV-1 γ34.5 to the nonenzymatic PP1α cofactor GADD34, it is likely that salubrinal also disrupts γ34.5 targeting of PP1α during infection. Additionally, due to its modulation of the UPR, salubrinal may prevent neuronal cell toxicity during infection beyond its effect on viral replication. Promotion of translational arrest by salubrinal during the UPR has been shown to be neuroprotective and to improve long-term neurologic outcomes in models of traumatic brain injury (39). In clinical practice, acyclovir is in widespread use to directly inhibit HSV replication; however, its use has not improved long-term neurologic outcomes among survivors of CNS disease (40). Development of therapeutics for HSV disease in newborns will need to be created to both reduce viral replication and to improve long-term neurologic outcomes in this susceptible population.

Methods

Viruses and Cells. The HSV-1 strain 17+ mutant virus ΔPP1α that carries the Val178Glu and Phe180Leu substitutions in γ34.5 and the rescue virus where the wild-type sequence was restored, ΔPP1α-R, were kindly provided by David Leib, Dartmouth University, Lebanon, NH and are previously described (19).

Vero cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) plus 10% (vol/vol) FBS and 1% penicillin–streptomycin and were used for the propagation and titering of virus. Plaque titrations were performed by standard methods.

Murine HSV Disease Model. Animal care and use in this study were in accordance with institutional and NIH guidelines, and all studies were approved by the Northwestern University Animal Care and Use Committee. The mouse strains used have been previously described, including the 129S2 (WT) and IFNAR KO mice (41) on the 129S2 genetic background. Pups were inoculated at 7 d of age, which from an immunologic perspective corresponds most closely to humans at birth (42). Virus was diluted to the appropriate dose in each experiment with PBS containing 1% inactivated calf serum and 0.1% glucose (PBS–GCS).

For the model of newborn disseminated HSV disease, neonatal mice were inoculated intraperitoneally with 10^4 pfu in 100 μL total volume of either ΔPP1α or ΔPP1α-R. Infected mice were monitored twice daily for signs of disease, and those displaying symptoms of advanced disease including lethargy, seizure, ataxia, and hunched posture were immediately killed, and the organs were collected for titering. Tissues were weighed, homogenized in DMEM with 5% (vol/vol) inactivated calf serum and 1% ciprofloxacin, and sonicated.

For the model of HSV encephalitis, newborn mice were inoculated intracranially with 10^2 pfu of either ΔPP1α or ΔPP1α-R. Infections of 8- to 10-wk-old adult mice were included for comparison with newborn infections, with an inocula of 10^4 pfu. A positive displacement syringe with a 26-gauge needle and a needle guard was used to inoculate 5 μL total volume into the brain. The needle was placed in the approximate region of the hippocampus, equidistant between the lambda and bregma through the left parietal bone lateral to the sagittal suture. Intracranially infected mice were similarly monitored twice daily and killed for viral titering. All statistical analyses were performed using Prism 5.01 (GraphPad Software). Kaplan–Meier survival statistical analysis was performed using the log-rank (Mantel–Cox) test. Comparisons of viral titers between different groups of mice were done by Student's *t* test, using log-transformed values.

Immunoblots. HeLa and Neuro2a cells were inoculated at a multiplicity of infection (MOI) of 5 on a rocking platform for 2 h at 37 °C. Infected cells were incubated until either 6 or 24 h postinfection, at which point they were collected for immunoblot analysis. Tissue samples were whole-organ homogenates from neonatal and adult mice. Western blots were performed on cell lysates using a 1:1,000 dilution of antibodies against total eIF2α (Cell Signaling), phosho-eIF2α (Ser51) (Cell Signaling), PKR (Abcam), PPP1R15B (Abcam), GADD34 (Abcam), and a 1:10,000 dilution of the anti-GAPDH antibody (Abcam) as a loading control. Blots were visualized using the LI-COR Odyssey system. Statistical analysis of densitometry was performed using a one-way ANOVA and the Holm–Sidak's multiple comparison test.

In Vitro Infections and Drug Treatment. HeLa cells were infected at an MOI of 0.001 with the rescue virus ΔPP1α-R suspended in a 1-mL volume of PBS–GCS with either the indicated concentration of salubrinal (Sigma) in DMSO or the equivalent volume of DMSO vehicle. Cells were infected on a rocking platform for 2 h, and the inoculum was replaced with fresh media containing either salubrinal or DMSO. Cells were collected and assayed for viral replication at the indicated time points.

Organotypic Brain Slice Cultures. Brain slice cultures were prepared from either 7-d-old neonatal mice or 8- to 10-wk-old adult mice. Mice were euthanized, and brains were rapidly removed into ice-cold slicing media [HBSS (Gibco) containing 6 mg/mL glucose equilibrated in 95% (vol/vol) O_2, 5% (vol/vol) CO_2]. Using aseptic technique, the frontal cortex and cerebellum were removed, and 400-μm coronal sections were made with a Vibratome through regions of the cerebrum containing the hippocampus and thalamus. Brain slices were positioned on a semiporous membrane insert (Millipore), following previously described methods (43). Membranes with brain slices were placed in six-well plates with 1.6 mL of filter culture media [50% (vol/vol) minimal essential media, 25% (vol/vol) HBSS, 25% (vol/vol) heat-inactivated horse serum, 0.044% $NaHCO_3$, 2 mM glutamine, 10 U/mL penicillin]. Immediately after establishment of the ex vivo culture, slices were infected with 10^6 pfu of the ΔPP1α-R rescue virus in 1 mL of PBS–GCS. Viral inoculum contained the indicated concentration of salubrinal (Sigma) or the equivalent volume of DMSO as a vehicle control. After 2 h, the inoculum was aspirated and replaced with fresh media containing either salubrinal or DMSO. Cultures were maintained in a humidified incubator at 5% (vol/vol) CO_2 and 37 °C and collected at 24, 48, or 72 h for viral titering.

ACKNOWLEDGMENTS. We thank Nanette Susmarski for providing cell culture expertise; Sarah Kopp for her help in establishing the brain slice cultures; the laboratory of Richard Miller at Northwestern University for help with brain slice cultures; and the laboratory of David Leib at Dartmouth University for providing various virus constructs. This work was supported by National Institutes of Health Grants T32AI060523 and F30AI116106 (to D.R.W.) and R01CA021776 (to R.L.).

1. Pinninti SG, Kimberlin DW (2013) Neonatal herpes simplex virus infections. *Pediatr Clin North Am* 60(2):351–365.
2. Kimberlin DW, et al.; National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group (2001) Natural history of neonatal herpes simplex virus infections in the acyclovir era. *Pediatrics* 108(2):223–229.
3. Langenberg AG, Corey L, Ashley RL, Leong WP, Straus SE; Chiron HSV Vaccine Study Group (1999) A prospective study of new infections with herpes simplex virus type 1 and type 2. *N Engl J Med* 341(19):1432–1438.
4. Bernstein DI, et al. (2013) Epidemiology, clinical presentation, and antibody response to primary infection with herpes simplex virus type 1 and type 2 in young women. *Clin Infect Dis* 56(3):344–351.
5. Jones CA, Raynes-Greenow C, Isaacs D; Neonatal HSV Study Investigators and Contributors to the Australian Paediatric Surveillance Unit (2014) Population-based surveillance of neonatal herpes simplex virus infection in Australia, 1997–2011. *Clin Infect Dis* 59(4):525–531.
6. Chou J, Chen JJ, Gross M, Roizman B (1995) Association of a M(r) 90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF-2 alpha and premature shutoff of protein synthesis after infection with gamma 134.5- mutants of herpes simplex virus 1. *Proc Natl Acad Sci USA* 92(23):10516–10520.
7. He B, Gross M, Roizman B (1997) The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. *Proc Natl Acad Sci USA* 94(3):843–848.
8. Leib DA, Machalek MA, Williams BR, Silverman RH, Virgin HW (2000) Specific phenotypic restoration of an attenuated virus by knockout of a host resistance gene. *Proc Natl Acad Sci USA* 97(11):6097–6101.
9. Cheng G, Gross M, Brett ME, He B (2001) AlaArg motif in the carboxyl terminus of the gamma(1)34.5 protein of herpes simplex virus type 1 is required for the formation of a high-molecular-weight complex that dephosphorylates eIF-2alpha. *J Virol* 75(8): 3666–3674.
10. Verpooten D, Ma Y, Hou S, Yan Z, He B (2009) Control of TANK-binding kinase 1-mediated signaling by the gamma(1)34.5 protein of herpes simplex virus 1. *J Biol Chem* 284(2):1097–1105.
11. Orvedahl A, et al. (2007) HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe* 1(1):23–35.
12. Tallóczy Z, et al. (2002) Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. *Proc Natl Acad Sci USA* 99(1):190–195.
13. Wilcox DR, Wadhwani NR, Longnecker R, Muller WJ (2015) Differential reliance on autophagy for protection from HSV encephalitis between newborns and adults. *PLoS Pathog* 11(1):e1004580.
14. Cheng G, Feng Z, He B (2005) Herpes simplex virus 1 infection activates the endoplasmic reticulum resident kinase PERK and mediates eIF-2alpha dephosphorylation by the gamma(1)34.5 protein. *J Virol* 79(3):1379–1388.
15. McGeoch DJ, Barnett BC (1991) Neurovirulence factor. *Nature* 353(6345):609.
16. He B, Chou J, Liebermann DA, Hoffman B, Roizman B (1996) The carboxyl terminus of the murine MyD116 gene substitutes for the corresponding domain of the gamma(1) 34.5 gene of herpes simplex virus to preclude the premature shutoff of total protein synthesis in infected human cells. *J Virol* 70(1):84–90.
17. Zhang C, et al. (2008) A conserved domain of herpes simplex virus ICP34.5 regulates protein phosphatase complex in mammalian cells. *FEBS Lett* 582(2):171–176.
18. Verpooten D, et al. (2009) Dephosphorylation of eIF2alpha mediated by the gamma134.5 protein of herpes simplex virus 1 facilitates viral neuroinvasion. *J Virol* 83(23):12626–12630.
19. Radtke K, et al. (2013) Inhibition of the host translation shutoff response by herpes simplex virus 1 triggers nuclear envelope-derived autophagy. *J Virol* 87(7):3990–3997.
20. Chou J, Roizman B (1992) The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc Natl Acad Sci USA* 89(8):3266–3270.
21. Meurs E, et al. (1990) Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell* 62(2):379–390.
22. Balachandran S, et al. (2000) Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity* 13(1):129–141.
23. Patel RC, Sen GC (1998) PACT, a protein activator of the interferon-induced protein kinase, PKR. *EMBO J* 17(15):4379–4390.
24. Ito T, Yang M, May WS (1999) RAX, a cellular activator for double-stranded RNA-dependent protein kinase during stress signaling. *J Biol Chem* 274(22):15427–15432.
25. Boyce M, et al. (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. *Science* 307(5711):935–939.
26. Bryant KF, et al. (2008) ICP34.5-dependent and -independent activities of salubrinal in herpes simplex virus-1 infected cells. *Virology* 379(2):197–204.
27. Chen SF, et al. (2004) Seizure, neuron loss, and mossy fiber sprouting in herpes simplex virus type 1-infected organotypic hippocampal cultures. *Epilepsia* 45(4):322–332.
28. Dionne KR, Leser JS, Lorenzen KA, Beckham JD, Tyler KL (2011) A brain slice culture model of viral encephalitis reveals an innate CNS cytokine response profile and the therapeutic potential of caspase inhibition. *Exp Neurol* 228(2):222–231.
29. Braun E, et al. (2006) Neurotropism of herpes simplex virus type 1 in brain organ cultures. *J Gen Virol* 87(Pt 10):2827–2837.
30. Bolovan CA, Sawtell NM, Thompson RL (1994) ICP34.5 mutants of herpes simplex virus type 1 strain 17syn+ are attenuated for neurovirulence in mice and for replication in confluent primary mouse embryo cell cultures. *J Virol* 68(1):48–55.
31. Chou J, Kern ER, Whitley RJ, Roizman B (1990) Mapping of herpes simplex virus-1 neurovirulence to gamma 134.5, a gene nonessential for growth in culture. *Science* 250(4985):1262–1266.
32. Kuma A, et al. (2004) The role of autophagy during the early neonatal starvation period. *Nature* 432(7020):1032–1036.
33. Siekierka J, Manne V, Ochoa S (1984) Mechanism of translational control by partial phosphorylation of the alpha subunit of eukaryotic initiation factor 2. *Proc Natl Acad Sci USA* 81(2):352–356.
34. Wek SA, Zhu S, Wek RC (1995) The histidyl-tRNA synthetase-related sequence in the eIF-2 alpha protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. *Mol Cell Biol* 15(8):4497–4506.
35. Kollmann TR, Levy O, Montgomery RR, Goriely S (2012) Innate immune function by Toll-like receptors: Distinct responses in newborns and the elderly. *Immunity* 37(5): 771–783.
36. Banisadr G, Podojil JR, Miller SD, Miller RJ (2015) Pattern of CXCR7 gene expression in mouse brain under normal and inflammatory conditions. *J Neuroimmune Pharmacol* PMID:25997895.
37. Mithal DS, Ren D, Miller RJ (2013) CXCR4 signaling regulates radial glial morphology and cell fate during embryonic spinal cord development. *Glia* 61(8):1288–1305.
38. Zheng LS, et al. (2014) Mechanisms for interferon-α-induced depression and neural stem cell dysfunction. *Stem Cell Rep* 3(1):73–84.
39. Rubovitch V, et al. (2015) The neuroprotective effect of salubrinal in a mouse model of traumatic brain injury. *Neuromolecular Med* 17(1):58–70.
40. Kimberlin DW, et al.; National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group (2001) Safety and efficacy of high-dose intravenous acyclovir in the management of neonatal herpes simplex virus infections. *Pediatrics* 108(2):230–238.
41. Müller U, et al. (1994) Functional role of type I and type II interferons in antiviral defense. *Science* 264(5167):1918–1921.
42. Adkins B, Leclerc C, Marshall-Clarke S (2004) Neonatal adaptive immunity comes of age. *Nat Rev Immunol* 4(7):553–564.
43. Stoppini L, Buchs PA, Muller D (1991) A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* 37(2):173–182.