1 **Encephalitic alphaviruses exploit caveolae-mediated transcytosis at the blood-brain** 2 **barrier for CNS entry**

3 Hamid Salimi¹, Matthew D. Cain¹, Xiaoping Jiang¹, Robyn A. Roth¹, Wandy Beatty¹, Chengqun Sun²,
4 William B. Klimstra², Jianghui Hou¹, Robyn S. Klein^{1,3,4}

5 ¹Department of Medicine, Washington University School of Medicine, St. Louis, MO

6 ²Department of Immunology and Center for Vaccine Research², University of Pittsburgh, Pittsburgh, PA

7 ³Department of Pathology & Immunology, Washington University School of Medicine, St. Louis, MO

8 ⁴Department of Neuroscience, Washington University School of Medicine, St. Louis, MO

9

10 Correspondence should be addressed to R.S.K. (rklein@wustl.edu).

11 Robyn S. Klein, M.D., Ph.D.

12 Washington University School of Medicine

13 Departments of Internal Medicine, Neurobiology,

14 Pathology & Immunology

15 Campus Box 8051

16 660 S. Euclid Ave

17 St. Louis, MO 63110

18 Phone: (314) 286-2140

19 Fax: (314) 362-9230

20 rklein@wustl.edu

21 **Abstract**

22 Venezuelan and Western equine encephalitis viruses (VEEV and WEEV) invade the CNS early during
 23 infection, via neuronal and hematogenous routes (1, 2). While viral replication mediates host-shut off,
 24 including expression of type I interferons (IFN) (3, 4), few studies have addressed how alphaviruses
 25 gain access to the CNS during established infection or the mechanisms of viral crossing at the blood-
 26 brain barrier (BBB). Here, we show that hematogenous dissemination of VEEV and WEEV into the
 27 CNS occurs via caveolin (Cav)-1-mediated transcytosis (Cav-MT) across an intact BBB, which is
 28 impeded by IFN and inhibitors of RhoA GTPase. Use of reporter and non-replicative strains also
 29 demonstrates that IFN signaling mediates viral restriction within cells comprising the neurovascular unit
 30 (NVU), differentially rendering brain endothelial cells, pericytes and astrocytes permissive to viral
 31 replication. Transmission and immunoelectron microscopy revealed early events in virus internalization
 32 and Cav-1-association within brain endothelial cells. Cav-1-deficient mice exhibit diminished CNS
 33 VEEV and WEEV titers during early infection, whereas viral burdens in peripheral tissues remained
 34 unchanged. Our findings show that alphaviruses exploit Cav-MT to enter the CNS, and that IFN
 35 differentially restricts this process at the BBB.

36

37 **Importance**

38 VEEV, WEEV and EEEV are emerging infectious diseases in the Americas, and they have caused
 39 several major outbreaks in the human and horse population during the past few decades. Shortly after
 40 infection, these viruses can infect the CNS, resulting in severe long-term neurological deficits or death.
 41 Neuroinvasion has been associated with virus entry into the CNS directly from the blood-stream,
 42 however the underlying molecular mechanisms have remained largely unknown. Here we demonstrate
 43 that following peripheral infection alphavirus augments vesicular formation/trafficking at the BBB and
 44 utilizes Cav-MT to cross an intact BBB, a process regulated by activators of Rho GTPases within brain
 45 endothelium. *In vivo* examination of early viral entry in Cav-1-deficient mice revealed significantly lower
 46 viral burdens than in similarly infected wild-type animals. These studies identify a potentially targetable
 47 pathway to limit neuroinvasion by alphaviruses.

48

49

50 Introduction

51 The central nervous system (CNS) is protected from pathogens by the blood brain barrier (BBB), an
 52 intercellular association of transmembrane junctional proteins between brain microvascular endothelial
 53 cells (BMECs), with associated pericytes, astrocytes and neurons that together comprise the
 54 neurovascular unit (NVU) (5). Neurotropic pathogens have evolved mechanisms to bypass or cross this
 55 barrier, including anterograde or retrograde transport along axons, destabilization of BBB junctional
 56 proteins, or passage through BMECs (6), the latter of which may involve intracellular transport within
 57 leukocytes via binding to intercellular adhesion molecule (ICAM)-1 (7). An additional mechanism may
 58 involve caveolae, flask-shaped plasma membrane invaginations within BMECs that are important for
 59 cell metabolism, signal transduction, and the transcytosis of large proteins (8). BBB formation of
 60 caveolae, which contain the major structural protein caveolin-1 (Cav-1), is limited by the major facilitator
 61 superfamily domain-containing protein 2a (Mfsd2a), which is exclusively expressed on BMECs and
 62 induced by pericytes (9). Stabilization of junctional proteins and caveolae within BMECs is additionally
 63 regulated by the small Rho GTPases, including the Ras homolog gene family, member A (RhoA), and
 64 Ras-related C3 botulinum toxin substrate (Rac)-1 (10). While many viruses have evolved to interact
 65 with Rho GTPases to increase their entry and replication within target cells (11, 12), some neurotropic
 66 viruses, including retroviruses and flaviviruses, may compromise BBB permeability via GTPase-
 67 mediated alterations of junctional proteins (13–16). For these viruses, neuroinvasion coincides with
 68 BBB instability. In contrast, encephalitic alphaviruses, including Venezuelan, Western and Eastern
 69 equine encephalitis viruses (VEEV, WEEV and EEEV, respectively), can enter the CNS directly from
 70 the bloodstream via unknown mechanisms (17, 18).

71 VEEV, WEEV, EEEV naturally cycle between mosquitoes and birds (EEEV and WEEV),
 72 mosquitoes and rodents (VEEV enzootic cycle), or mosquitoes and horses (VEEV epizootic cycle), and

are all widely distributed in North, Central, and South America (19). Human infection can progress rapidly to encephalitis with fatality rates of 1-75%, depending on the strain. Of the three, VEEV is considered the most important zoonotic pathogen with several reported outbreaks in South and Central Americas, the latter of which have spread to North America. Although the number of human cases reported is small, the possibility for disease emergence is high due to expansion and spread of mosquito vectors (20). Despite the epidemic potential of VEEV and the high morbidity and/or case fatality rates of EEEV and WEEV, there are no approved vaccines or therapeutics for humans. Insight into the cell-intrinsic and -extrinsic processes by which the host limits alphavirus infections and minimizes virus- and immune-induced injury is essential for developing strategies to contain virus dissemination and disease. While early studies suggested that VEEV enters the CNS via anterograde transport along peripheral nerves after cutaneous inoculation (1), recent findings, however, emphasize that VEEV may cross the BBB via unknown mechanisms (2, 21).

In this study, we show that peripherally inoculated virulent strains of VEEV and WEEV enter the CNS from the blood-stream as free virions through an intact BBB. While VEEV and WEEV interact, enter and traverse brain endothelium *in vivo*, virus replication within brain microvascular endothelial cells (BMECs) and pericytes is inhibited by type I IFN (IFN) signaling. Consistent with this, reporter and non-replicative strains of VEEV and WEEV were observed to first replicate within astrocytes and neurons, respectively, suggesting that alphaviruses cross the BBB without replication in BMECs or pericytes. Using an *in vitro* transcytosis assay, alphaviruses were found to utilize caveolin-mediated transcytosis (Cav-MT), which is directly regulated by small Rho GTPases, and, notably, IFN. Transmission and immunoelectron microscopy revealed *in vivo* virus encounter and entry at the BBB, with detection of VEEV within caveolin-1-expressing BMECs. Importantly, deficiency in *Cav-1* significantly delayed alphavirus neuroinvasion during early infection, highlighting the important role of

96 caveolae in CNS entry. Together, these data suggest that IFNs regulate the CNS entry of encephalitic
97 alphaviruses via direct and indirect mechanisms.
98

99 Results

100 **Alphavirus neuroinvasion occurs prior to BBB disruption.** To address mechanisms of alphavirus
 101 neuroinvasion in susceptible hosts, we infected mice with enzootic VEEV ZPC738 (VEEV) and
 102 epizootic WEEV McMillan (WEEV) strains of alphaviruses (22, 23). Wild-type mice infected with VEEV
 103 and WEEV via footpad (f.p.) inoculation exhibited detectable virus replication simultaneously in both
 104 fore- and hindbrain regions at 1- and 3-days post-infection (dpi), respectively (Fig. 1A and B). Similar
 105 results were observed in the brainstem and spinal cord (Fig. S1A-D). Consistent with prior studies, the
 106 olfactory bulb exhibited higher VEEV titers at early time-points (1). Viral titers plateaued at 10^7 to 10^8
 107 pfu/g of tissue by 3-4 dpi (VEEV) and 4-5 dpi (WEEV). Significant alterations in BBB permeability
 108 occurred at plateau viral loads, with VEEV infection leading to higher permeability in all brain regions as
 109 compared to WEEV (Fig. 1C and D). Peak BBB permeability in the brainstem and spinal cord also
 110 occurred at time-points coinciding with peak viral loads (Fig. S1C and D). We also examined direct
 111 effects of virus on BBB integrity using an *in vitro* BBB model, in which primary murine BMECs are
 112 cultured on transwell inserts over primary murine astrocytes in the bottom chamber (Fig. 1E). In this
 113 model, barrier integrity is assessed by measuring transendothelial electrical resistance (TEER) between
 114 the transwell chambers. While control cultures treated with TNF- α (100 ng/ml) displayed decreased
 115 TEER at all time-points, addition of virus to either BMECs or astrocytes had no effect on TEER (Fig. 1F
 116 and G). Together, our observations from *in vivo* and *in vitro* experiments suggest that alphavirus
 117 neuroinvasion occurs in the presence of an intact BBB.

118

119 **Hematogenous route of neuroinvasion is not exclusive to the circumventricular organs (CVOs).**
 120 Viral neuroinvasion across an intact BBB could occur via virus replication within cellular constituents of
 121 the NVU. To assess viral permissivity of NVU cells, we examined *in vitro* alphavirus infection of isolated

murine cells and performed multistep growth curve analyses after infection with either VEEV (Fig. S2A-C) or WEEV (Fig. S2E-G). While all cell types were permissive to both viruses, slopes of the curves differed; thus, virus replication plateaued as early as 6-12 hours post-infection (hpi) in astrocytes, while pericytes and BMECs required 12-24 hpi and 24-48 hpi, respectively, to reach plateau levels. To validate these findings in murine BMECs, the experiment was performed using the human brain endothelial cell line, hCMEC/D3, which displayed similar results (Fig S1H and L). These data suggest that differential restriction of alphavirus replication may occur at the NVU. To examine this *in vivo* during early viral neuroinvasion, we infected mice with 10^6 pfu of either VEEV-eGFP or WEEV-eGFP via intravenous (i.v.) injection, followed by immunohistochemical (IHC) detection of GFP within brain tissues. Given the two-day delay in detection of WEEV compared with VEEV within the CNS (Fig. 1) CNS tissues of VEEV-infected mice were examined at 16 hpi, while WEEV-infected mice were examined at 48 hpi. Analyses of brains revealed multiple foci of viral replication throughout the brain for both viruses, suggesting hematogenous routes of CNS entry. While some of the entry sites overlapped with areas consistent with CVOs (Fig. 2A and B; arrows) (2), others were located within cortical and cerebellar areas that are distant from these structures (Fig. 2A and B; arrow heads). In VEEV-infected mice, GFP expression was detected in both NeuN⁺ neurons and S100- β ⁺ astrocytes (Fig. 2C). In contrast, WEEV-infected animals exhibited GFP expression exclusively within neurons (Fig. 2D). Notably, neither reporter strain led to early viral replication within BMECs or pericytes (Fig. 2C and D), which is consistent with their diminished permissivity *in vitro* compared with astrocytes. Together, these data suggest that cells of the NVU are differentially susceptible to alphavirus infection *in vivo* and that, upon viremia, alphaviruses invade the CNS from the bloodstream through multiple entry sites that are not exclusive to CVOs.

145 **IFNAR signaling differentially restricts alphavirus infection at the BBB.** Given our results
 146 demonstrating differential *in vivo* replication of alphaviruses within cells of the NVU, we hypothesized
 147 that robust post-entry restriction may be imposed by innate immune responses within BMECs and
 148 pericytes. To address this, WT and *Ifnar*^{-/-} mice were infected with VEEV-eGFP (100 pfu) via f.p.
 149 inoculation, and brain tissues were examined at 1 dpi for GFP expression. Notably, *Ifnar*^{-/-} mice
 150 succumb to VEEV-eGFP by ~30 hr post infection (Fig. S3A), while WT animals have undetectable brain
 151 infection using IHC at this time-point (Fig S3D). Thus, to allow comparisons at similar viral burdens (Fig.
 152 S3C), we also evaluated brain tissues of WT mice at 3 dpi. VEEV infection was limited to S100-β⁺
 153 astrocytes and NeuN⁺ neurons in WT animals (Fig. 3A and Fig. S4A), whereas similar infection in *Ifnar*^{-/-}
 154 animals led to GFP expression within BMECs and pericytes both in the cortex (Fig. 3B) and
 155 cerebellum (Fig. S4B). Next, we assessed cellular tropism in the context of WEEV-eGFP (1000 pfu)
 156 infection. Although mortality was significantly increased in WEEV-eGFP-infected *Ifnar*^{-/-} mice compared
 157 with similarly infected WT animals, *Ifnar*^{-/-} mice survive WEEV infection up to 5-6 dpi (Fig. S3B), and
 158 viral infection was undetectable in brain tissues of either WT or *Ifnar*^{-/-} mice at 1 dpi (Fig. S3E). Thus,
 159 brain tissues were examined at 3 dpi for both genotypes. Similar to our observations with VEEV, WT
 160 mice infected with WEEV exhibited no GFP expression in BMECs or pericytes, with infection detected
 161 only in neurons (Fig. 3C and Fig. S4C). However, *Ifnar*^{-/-} mice additionally exhibited GFP expression
 162 within cortical (Fig. 3D) and cerebellar (Fig. S4D) astrocytes.

163 As the observed changes in cellular tropism could be due a higher titer of viremia in *Ifnar*^{-/-} versus WT
 164 mice, *Ifnar*^{-/-} mice were infected with 10⁶ pfu/ml of a non-replicative (NR) replicon derived from VEEV-
 165 eGFP (24); a physiologically relevant dose that is equivalent to the level of viremia observed in WT
 166 mice after f.p. inoculation (Fig. S4F). Following cellular entry, NR-VEEV-eGFP undergoes RNA
 167 replication and produces reporter genes products, but no progeny virus, due to lack of structural genes

(24). Similar to our results with WT VEEV-eGFP, WT animals infected with NR-VEEV-eGFP had no infection of BEMCs or pericytes (Fig. 3E), whereas *Ifnar*^{-/-} mice exhibited extensive infection of these cell types in multiple brain regions, including the cortex (Fig. 3F) and cerebellum (Fig. S4E). These findings support the notion that alphavirus infection is differentially restricted at the NVU by IFNAR signaling, which prevents CNS entry by blocking active virus replication within BMECs.

Ultrastructural analysis reveals alphavirus interaction and entry at the BBB. To further characterize virus-cell interactions at the BBB *in vivo*, we performed transmission electron microscopy (TEM) of CNS tissues derived from VEEV-infected WT and *Ifnar*^{-/-} mice. TEM analysis of *Ifnar*^{-/-} mice, which develop high level viremia (Fig. S4F), revealed 70 nm spherical structures with electron dense cores, consistent with virions (25), attached to the luminal surface of cortical microvessels (Fig. 4A). Remarkably, immunogold labeling using antibodies against the VEEV E2 glycoproteins detected viral particles inside cortical BMECs of WT mice (Fig. 4B). Since WT BMECs do not support VEEV replication *in vivo* (Fig. 3A), detection of E2 glycoproteins likely represents an intact virion rather than intracellular protein expression. Based on these observations, we hypothesized that VEEV may cross the BBB as free particles. To examine this, mice were infected with NR-VEEV-eGFP via *i.v.* injection. At 1 dpi, we detected infection of astrocyte-like cells in multiple brain regions, including cortex and cerebellum (Fig. 4C and D), suggesting that VEEV can cross the BBB as free virions, likely via a transcytosis mechanism.

Alphavirus crosses brain endothelial cells via caveolae-mediated transcytosis. Based on our TEM data (Fig. 4A and B), we hypothesized that alphaviruses may exploit BMEC endocytic machinery

for entry across the BBB. Contrary to peripheral tissues, the rate of transcytosis and numbers of endocytic vesicles are relatively low in BMECs (9). However, exposure of BMECs to VEEV or WEEV (MOI 10) resulted in enhanced caveolae formation at the cell surface (Fig. 5A-D). We next quantified the number of caveolae-like structures in BMECs of VEEV-infected mice. Similar to our *in vitro* data, we observed increased vesiculation in cytoplasm and at the luminal surface of BMECs in infected animals (Fig. 5E-G). In addition, virus infection induced membrane ruffling in BMECs of infected mice (Fig. 5H). These observations suggest a possible mechanism for viral transcytosis. To address this, we utilized a transcytosis assay in which virus is added to the top chambers of transwell inserts for 60 min, followed by removal of inserts and assessment of infectious virions in the bottom chamber (Fig. 6A). As the replication time of VEEV in BMECs is approximately 3-6 h (Fig. S5A), virus detected in the bottom chamber is unlikely due to replication, which was confirmed via assessment of GFP expression in bottom chamber astrocytes after transcytosis of NR-VEEV-eGFP virus (Fig. 6B). TEM evaluation of virus transcytosis across BMECs additionally identified virion-like structures within endosomes, characteristic of caveolae (26). Virion-containing endosomes were detected at various stages of transcytosis, i.e. early endosomes, multi-vesicular bodies and exocytic vesicles (Fig. 6C-E). Together, these data suggest that VEEV has the ability to cross endothelial cell barrier via a transcytosis mechanism.

Transcytosis may utilize various pathways, including macropinocytosis, and clathrin- or caveolae-mediated endocytosis (CME & Cav-ME, respectively) (10, 27), which may be interrogated via use of inhibitors including amiloride hydrochloride (macropinocytosis); filipin, nystatin and cholesterol oxidase (Cav-ME), and chlorpromazine (CME). Compared with untreated BMECs or hCMEC/D3, the percentage of transcytosis for both VEEV and WEEV was significantly reduced in the presence of inhibitors of Cav-ME, but not CME or macropinocytosis (Fig. 6F-I). Notably, none of the inhibitors

213 affected viral infectivity or barrier integrity (Fig. S5B-E). In accordance with the key role of caveolin-1 in
 214 caveolae formation, *Cav-1^{-/-}* BMECs displayed diminished virus transcytosis as compared to WT cells
 215 (Fig. 6J). These results were further verified using freeze fracture electron microscopy, wherein we
 216 identified VEEV particles (8 nm gold particle; orange arrow) co-localized with Cav-1⁺ (16 nm gold
 217 particles, white arrow) (Fig. 6K). In addition, lung endothelial cells exhibited significantly reduced virus
 218 transcytosis compared to BMECs (Fig. 6L), suggesting specificity of alphavirus transcytosis for BMECs.

219 Caveolae formation is enhanced by the small Rho GTPase RhoA, which is inhibited by Rac1
 220 activity (28, 29). Consistent with this, H-1152 (Rho-kinase inhibitor) significantly reduced VEEV
 221 transcytosis across BMECs, while Z62954982 (Rac-1 inhibitor) had no significant effect on virus
 222 transmigration (Fig. 6M). However, treatment with IFN- α or IFN- β , which has been shown to promote
 223 BBB tight junction integrity via activation of Rac-1 (15), significantly reduced VEEV transcytosis,
 224 suggesting direct effects of IFNAR signaling on Cav-MT (Fig. 6N). Together, these results indicate that
 225 VEEV and WEEV exploit Cav-1-MT to cross an intact BBB and that this may be regulated by IFN.

226

227 **Caveolin-1 contributes to alphavirus neuroinvasion *in vivo*.** To validate the role of caveolin-1 in
 228 early alphavirus neuroinvasion *in vivo*, we infected WT and *Cav-1^{-/-}* mice with either VEEV (10 pfu) or
 229 WEEV (1000 pfu) via f.p. infection and examined CNS viral burdens at 1 and 3 dpi. Both VEEV and
 230 WEEV-infected *Cav-1^{-/-}* mice exhibited significantly reduced viral titers in the cortex and cerebellum at 1
 231 dpi and 3 dpi, respectively, as compared to similarly infected WT animals, whereas viral burden in the
 232 serum and spleen were indistinguishable between the two genotypes (Fig. 7A and B). Importantly, no
 233 differences in CNS viral loads were detected in intracranial alphavirus-infected WT versus *Cav-1^{-/-}* mice
 234 (Fig. S6A and B), indicating no effect of Cav-1-deficiency on viral replication within the CNS. These

235 results indicate that caveolin-1 critically contributes to alphavirus neuroinvasion after peripheral
236 infection.

237 Discussion

238 Using *in vitro* and *in vivo* approaches, we examined early events during alphavirus
239 neuroinvasion. We found that VEEV and WEEV can enter the CNS via hematogenous dissemination
240 across an intact BBB, without viral replication within BMECs or pericytes, leading to productive infection
241 of CNS resident cells. Differential restriction of viral infection within cellular constituents of the NVU is
242 mediated by IFNAR signaling, with replication in BMECs and pericytes (VEEV), and astrocytes (WEEV)
243 only observed in *Ifnar*^{-/-} mice. VEEV and WEEV entry across the BBB occurs via Cav-MT, which is
244 impeded by Rho kinase inhibitor and IFN, the latter likely via activation of Rac1 (15, 16). Immuno-EM
245 demonstrated alphavirus interaction, internalization and Cav-1 association within BMECs both *in vitro*
246 and *in vivo*. Consistent with this, Cav-1-deficiency reduced alphavirus transcytosis *in vitro* and led to
247 reduced titers of VEEV or WEEV in the CNS during early infection *in vivo*. Taken together, these data
248 suggest that innate immune signaling regulates alphavirus neuroinvasion and replication at the NVU.

249 The CNS entry of alphaviruses following peripheral infection has been shown to occur via
250 multiple routes. Early studies in mice indicated that VEEV may reach the brain via retrograde transport
251 along peripheral nerves (1), whereas, more recently VEEV and other encephalitic alphaviruses have
252 been shown to enter the CNS directly from the bloodstream (2, 17, 30). In our study, peripheral
253 infection of mice with virulent strains of VEEV and WEEV resulted in detectable viral loads at time-
254 points that precede BBB disruption (Fig. 1), and neither VEEV nor WEEV induced alterations in TEER
255 across an *in vitro* BBB. These results indicate that alphaviruses cross the BBB shortly after infection
256 without affecting barrier integrity. Hematogenous routes of neuroinvasion may include viral passage
257 through the CVOs, which lack specializations that comprise the BBB (2). Our study demonstrated that
258 shortly after *i.v.* infection (16hrs), foci of alphavirus infection are observed simultaneously in cortical and
259 cerebellar locations distant from the CVOs, suggesting that CNS entry is not exclusive to these

structures. Notably, BBB breakdown occurs at later time-points, when viral loads have peaked, likely due to induction of inflammatory responses and infiltration of immune cells into the CNS (21, 31, 32).

Pathogens, including viruses, may cross the BBB via direct infection of brain endothelium (33, 34). Use of reporter and non-replicative strains of VEEV and WEEV during *in vivo* infection, however, indicated lack of infection within BMECs (Fig. 3), suggesting that active virus replication in brain endothelium does not contribute to CNS entry. VEEV has a broad tissue tropism and can infect many cell types, including macrophages and dendritic cells (24). Infected leukocytes may act as Trojan horses and introduce viral particles into the CNS upon infiltration. However, leukocyte interaction and extravasation at the BBB require elevated expression of cell adhesion molecules (CAMs) on brain endothelium, which does not occur until 3dpi (21). Since we detected significant viral loads throughout the CNS by 1 dpi (Fig. 1), it is unlikely that infected leukocytes are the initial source of virus in the CNS. Importantly, our data using NR-VEEV-eGFP did not detect viral infection within CNS infiltrating leukocytes, suggesting that virus can enter CNS as free virion and independent of leukocyte trafficking. Nonetheless, additional studies are required to precisely define the role of Trojan horse in alphavirus neuroinvasion.

Our TEM analysis demonstrated virus like particles attached to the lumen of brain endothelium. Given that viremia arises as early as 8 hr post VEEV infection, BMECs are likely among the first cell types exposed to virions. Such exposure may trigger innate immune responses (e.g. type I IFNs), which can influence intercellular communication at the NVU (35). Indeed, in the context of IFNAR deficiency, VEEV and WEEV replicate within BMECs and astrocytes, respectively. The lack of viral replication within these cellular constituents of the NVU in WT animals is likely due to paracrine effects of IFN that induce antiviral proteins, as has been recently reported for arthritogenic alphaviruses (36, 37). VEEV attachment to and internalization within BMECs was further confirmed via immuno-TEM, in which viral

283 E2 glycoproteins were detected within these cells, supporting the notion that alphaviruses may cross
284 the BBB via a transcytosis pathway.

285 Compared to peripheral tissues, the rate of transcytosis at the BBB is unusually low. However,
286 this rate may be augmented by increased Src kinase activity, which is mediated by a group of
287 pathologic and non-pathologic stimuli, including inflammatory mediators and immune cell interactions
288 with brain endothelium (38). Viruses may also increase vesicular trafficking, as has been observed in
289 peripherally-derived endothelial cells exposed to dengue virus (39). In our study we utilized both *in vitro*
290 assays and *in vivo* viral infection models, and observed that both VEEV and WEEV induce formation of
291 caveolae in BMECs. *In vivo*, VEEV infected mice exhibited increased vesiculation and membrane
292 ruffling within brain endothelial cells (Fig. 5). *In vitro* studies have shown that alphaviruses may induce
293 dramatic structural changes in the actin cytoskeleton, leading to the formation of filopodia-like
294 extensions in infected cells (40). Notably, expression of the viral non-structural protein (nsP)-1 alone is
295 sufficient to trigger formation of short extensions, which is dependent on its palmitoylation activity (41).
296 Consistent with this, ablation of nsP1 palmitoylation sites abolishes the ability of Semliki Forest virus
297 (SFV) to infect the brain in murine models (42). Elucidating molecular mechanisms underlying
298 alphavirus induced vesiculation in BMECs may reveal therapeutic targets against viral neuroinvasion,
299 and provide insights for BBB maintenance in the context of other neurological disorders induced by
300 increased transcytosis in brain endothelium (43–45).

301 Transcytosis of macromolecules across brain endothelium predominantly occurs via
302 mechanisms that utilize caveolae. During infectious diseases, interaction with caveolae allows
303 pathogens to escape lysosomal degradation and cross endothelial cell barriers (46, 47). While different
304 families of viruses have been shown to cross epithelial and endothelial cell barriers utilizing Cav-MT *in*
305 *vitro* (48–50), *in vivo* findings to support these data have been difficult to obtain, especially within the

brain where these events are rare. Our study utilized multiple approaches to demonstrate that neurotropic alphaviruses enter but do not replicate within brain endothelium, and that Cav-MT contributes to *in vivo* viral infection of the CNS. As peripherally infected *Cav-1^{-/-}* mice exhibit a delay in achievement of peak brain titers of VEEV and WEEV compared to WT animals, entry of virus in the absence of caveolae may rely on slower mechanisms of viral entry, such as retrograde transport along axons (1). Additionally, ablation of caveolin-1 results in upregulation of caveolin-independent pathways (51), which might explain how viral loads within CNS tissues derived from *Cav-1^{-/-}* mice quickly attain the levels observed in WT animals.

The signaling cascade underlying transcytosis was further elaborated using *in vitro* assays, wherein deficiency in caveolin-1 or depletion of membrane cholesterol significantly reduced VEEV and WEEV transcytosis across BMEC monolayer. Notably, virus transcytosis across brain endothelial cells was blocked by Rho kinase inhibitor (Fig. 6). The Rho family of GTPases, including RhoA, Rac-1 and Cdc42 are known to play a role in pathogen uptake and dissemination within the host (52–55). These proteins alternate between an active GTP-bound, and an inactive GDP-bound form, which triggers rearrangements in the actin cytoskeleton and cellular uptake of pathogens (56). Studies in flavivirus encephalitis have shown that IFN β acts in synergy with the TAM receptor Mertk to activate of Rac-1, which enhances BBB tight junction integrity, thereby preventing paracellular entry of virus (15, 16). In the current study, we discovered that type I IFN also prevents alphavirus transcytosis across BMECs. Thus, although the VEEV nsP2 and capsid protein inhibits IFN signaling via host-shut off (4), the lack of viral replication within BMECs may allow IFN signaling to limit viral entry likely via modulation of Rho GTPases (15).

In summary, mechanisms of encephalitic alphavirus neuroinvasion from the blood are complex and may depend on individual viral tropism for olfactory sensory neurons and cellular constituents of

329 the NVU, which provide avenues of entry that cross the BBB, respectively (24, 57). Neuroinvasion may
 330 also depend on innate immune mechanisms that exert virus and cell-specific effects on replication (4).
 331 Ablation of the olfactory route reduces but does not eliminate CNS entry of VEEV (1), suggesting each
 332 route of entry provides a relative contribution to alphavirus neuroinvasion. Further studies are needed
 333 to address how each process individually contributes to viral entry, and whether viral spread within the
 334 CNS relies on these pathways or on innate immune mechanisms that regulate intracellular viral
 335 trafficking and replication within permissive cells. Our data highlighted the critical role of Cav-MT in
 336 alphavirus neuroinvasion. Delineating molecular mechanisms involved in this process may reveal novel
 337 targets for potential therapeutic interventions against CNS infection.

338

339 MATERIALS AND METHODS

340 **Cells.** Baby hamster kidney (BHK-21) and African green monkey kidney (Vero) cells were maintained
341 in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and
342 100 µg/ml of penicillin and streptomycin. Primary murine brain microvascular endothelial cells
343 (BMECs), pericytes and astrocytes were isolated from cortical brain of C57BL/6J mice, and maintained
344 in culture as described previously (15, 58). The immortalized human cerebral microvascular endothelial
345 cell line hCMEC/D3 (59) was purchased from Millipore Sigma and maintained in Endothelial Cell
346 Growth Basal Medium-2 (EBM2, Lonza). Primary human astrocytes and murine lung endothelial cells
347 were purchased from ScienCell Research Laboratories and Cell Biologics, respectively. Cells were
348 maintained in the culture medium recommended by each company.

349

350 **Antibodies and reagents.** Rabbit polyclonal anti-S100-β (ab41548), rabbit monoclonal anti-Calbindin
351 (ab108404) and rabbit polyclonal anti-RFP (ab62341) antibodies (Abs) were purchased from Abcam
352 Biotechnology. Purified rat anti-mouse CD31 (550274, clone MEC 13.3) and guinea pig anti-NeuN
353 (ABN90P) polyclonal Abs were purchased from BD Biosciences and Millipore-Sigma, respectively.
354 Goat anti-PDGFR-β (AF1042) Ab was purchased from R&D Systems. The anti-VEEV E2 monoclonal
355 antibody was a kind gift from Dr. Michael Diamond (Washington University in St Louis). Amiloride
356 hydrochloride hydrate, filipin, cholesterol oxidase, nystatin, chlorpromazine hydrochloride, Z62954982
357 and fluorescein sodium salt were purchased from Sigma-Aldrich. (S)-Glycyl-H-1152 (hydrochloride)
358 was purchased from Cayman company. Mouse IFN-α and -β were obtained from PBL Assay Science.

359

Virus propagation, purification, and titration. All viruses used in this study including the non-replicative “replicon” strain of VEEV (NR-VEEV-eGFP), replication competent VEEV, McMillan (WEEV), and GFP reporter viruses (60) were generously provided by Dr. William Klimstra (University of Pittsburgh, Pittsburgh, PA). The parental VEEV and WEEV cDNAs were gifts to Dr. Klimstra from Dr. Scott Weaver, University of Texas Medical Branch at Galveston and Dr. Kenneth Olson, Colorado State University, respectively. The McMillan cDNA was modified by placing the entire virus coding region in a pBR322-based plasmid under control of a T7 bacteriophage promoter. Stocks of VEEV and WEEV viruses were generated as described previously (61). Briefly, BHK-21 cells were infected at a multiplicity of infection (MOI) of 0.1. Culture medium was replaced with fresh medium 4 hr later. Virus-containing supernatants were collected on the next day (~30 h post infection), cleared from cell debris by low-speed centrifugation and filtered through 0.22 µm filters. Virus particles were then concentrated by ultracentrifugation at 100,000 g for 2 h at 4°C through a cushion of 30% (wt/wt) sucrose in PBS. The virus pellet was re-suspended in PBS and stored in single use aliquots at -80°C. Titration of VEEV and WEEV were performed in BHK-21 and Vero cells, respectively.

374

Multi-step growth curves. Primary murine BMECs, astrocytes and pericytes were seeded in 24-well plates for 3-4 days till they reached confluence. At this point cells were exposed to MOIs of 0.01, 0.1, and 1 of VEEV and WEEV viruses. Culture medium was removed one hour post infection and cells were washed 4 times with 1 ml/well of PBS. The last wash was stored at -80°C, and later it was used in plaque assay to determine residual unbound virus remaining in each well. Culture supernatant was harvested at specified time points and virus titer was determined using plaque assay in BHK-21 (VEEV) and Vero cells (WEEV).

382

383 **Mice studies.** C57BL/6J wild-type and caveoline-1 knockout mice were purchased from Jackson
 384 Laboratory (Bar Harbor, ME). *Ifnar*^{-/-} mice were kindly provided by Dr. Michael Diamond (Washington
 385 University School of Medicine, St. Louis, MO). Animals were housed under pathogen-free conditions,
 386 and the experimental procedures were completed in accordance with the Washington University School
 387 of Medicine Animal Safety Committee. Male, 8- to 9-week-old mice were used in all *in vivo*
 388 experiments. Mice infections were performed by administrating virus either subcutaneous (SC),
 389 intravenous (IV) or intracranial (IC) injection, while the mice were under light ketamine anesthesia. For
 390 subcutaneous infection, mice were injected in the footpad with VEEV (10 pfu) and WEEV (1000 pfu) in
 391 50 µl PSB. Intracranial infections were performed by inoculating 10 pfu (VEEV) or 100 pfu (WEEV) of
 392 virus in 10 µl of PBS into the right cerebral hemisphere via a guided 29-gauge needle. Intravenous
 393 infections were achieved by administrating 2*10⁶ pfu of virus in 100 µl of PBS via retro-orbital injection.
 394 Mock infected animals received the same volume of PBS in each infection method and were
 395 considered as controls where needed.

396

397 **Measurement of viral burden in tissues.** To monitor the kinetic of virus spread *in vivo*, peripheral
 398 organs and CNS tissues were harvested from infected mice at specified time points. Prior to this, mice
 399 were perfused transcardially with 30 ml PBS. Tissues were homogenized in 500 µl PBS using MagNA
 400 Lyser (6000 rpm for 1 min) instrument, and virus titer in tissues homogenates was determined using
 401 plaque assay in either BHK-21 or Vero cells depending on virus. Alternatively, groups of mice were
 402 followed for survival assays.

403

404 ***In vivo* assessment of BBB permeability.** At given days post infection, mice received 100 µl of 100
405 mg/ml fluorescein sodium salt (NaFL) in PBS via intraperitoneal injection. When the salt has reached
406 equilibrium (45 min), blood samples were collected, and mice were perfused transcardially with 30 ml
407 PBS prior to collection of CNS tissues. Serum samples and tissue homogenates were treated with 2%
408 trichloroacetic acid overnight at 4°C to precipitate proteins. Supernatants were clarified from cellular
409 debris by centrifugation (4000 rpm for 20 min at 4°C) and diluted in equal volumes of borate buffer, pH
410 11 (Sigma- Aldrich). Concentration of NaFL in supernatants was determined by measuring fluorescence
411 emission at 538 nm using Synergy H1 microplate reader (BioTek Instruments, Inc.). Measurement
412 values were normalized to tissue weight and to NaFL plasma concentration in each mouse.

413

414 ***In vitro* BBB model and transcytosis assay.** An *in vitro* BBB model was generated as described
415 elsewhere (61). Briefly, BMECs were seeded on the apical side of fibronectin-coated inserts (BD
416 Falcon, 24-well, 3 µm pores). Concurrently, primary murine astrocytes were cultured in fibronectin-
417 coated 24-well plates. Two days later, when astrocytes reached confluent, BMECs inserts were moved
418 to astrocytes-containing plate. Astrocytes release growth factors that promote barrier formation
419 between BMECs. Culture medium was changed every 3 days. On day 7, hydrocortisone (550 nM),
420 CTP-cAMP (250 µM) and RO 20-1724 (17.5 µM) compounds were added to the cells in serum free
421 medium to further promote expression of tight junctions and barrier formation between BMECs. On the
422 following day, barrier integrity was assessed by measuring transendothelial electric resistance (TEER)
423 before cells were used for transcytosis assay. In these assays, BMECs on the top chamber are
424 exposed to different treatments for 1-2 h. Virus particles are then added at a MOI of 2 to BMECs on the
425 top chamber for an additional hour. At this point, BMEC inserts are removed and culture medium is
426 collected from the bottom chamber and is used in plaque assay to determine the number of virus

particles that have crossed the BMECs monolayer in the presence and absence of different inhibitors. Exposure of BMECs to alphavirus is limited to one hour to prevent active virus replication and release of viral progenies from the basolateral side.

Immunohistochemistry and confocal microscopy. Brain tissues were collected from infected mice at specified time points as indicated in each figure legend. Prior to tissue collection, mice were deeply anesthetized and perfused transcardially with 30 ml of PBS followed by 30 ml of 4% paraformaldehyde (PFA). Tissues were stored overnight in 4% PFA in PBS at 4°C, then transferred into two exchanges of 30% sucrose for 48 h, before they were embedded in O.C.T compound (Tissue-Tek). 10 µm cryostat brain sections were treated with proteinase K (5 µg/ml for 30 min at RT) for antigen retrieval. After 30 min incubation in blocking buffer, tissues sections were stained with primary antibodies to markers for astrocytes (S100 calcium-binding protein (S100)-β), pericytes (platelet-derived growth factor receptor beta/PDGFR-β), BMECs (CD31) and neurons (NeuN and calbindin). Sections were then incubated with appropriate secondary antibodies (Alexa Fluor 488, 555, and Dylight 650, all from Invitrogen) for 15 min in blocking buffer, followed by three washes in PBS. After antibody labelling, cells were counterstained with DAPI (D1306, Invitrogen). All images were obtained using confocal microscope (Carl Zeiss), and processed with ImageJ software (NIH).

Transmission- and immunogold electron microscopy. For TEM, mice underwent cardiac perfusion with 5 ml of PBS, followed by 20 ml of 1.5% glutaraldehyde and 1% PFA in 0.12 M sodium pyrophosphate buffer, while for immuno-EM studies, perfusion was performed with 5 ml of PBS and 20 ml of 4% paraformaldehyde in 0.12 M sodium pyrophosphate buffer. Brain tissues were then collected

and stored in fixative buffer overnight at 4°C and processed as previously described(61). For immuno-EM, ultrathin brain sections (70 nm) were stained with primary and gold-conjugated secondary antibodies on carbon-coated glass. Sections were viewed on a JEM-1400 transmission microscope (JEOL) at 80 KV with an AMT XR111 4k digital camera. For *in vitro* assays, monolayer of BMECs on Transwell inserts was infected with alphavirus at a MOI of 200. After 30 min, culture medium was replaced with fixative buffer (2% PFA and 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2) and incubated for 1 h at room temperature. Cells were then rinsed with sodium cacodylate buffer, and embedded in a thin layer of 2.5% agarose, followed by 1 h of fixation in 1% osmium tetroxide (Polysciences Inc.). After extensive wash with dH₂O, cells were stained with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 h, dehydrated through a series of ethanol concentrations in distilled water, and embedded in Eponate 12 resin (Ted Pella Inc.). Ultrathin sections were generated at 95 nm using UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), and stained with uranyl acetate and lead citrate. Electron micrographs were obtained using a transmission electron microscope JEOL 1200 EX (JEOL USA Inc., Peabody, MA) equipped with an AMT 8-megapixel digital camera and AMT Image Capture Engine V602 software (Advanced Microscopy Techniques, Woburn, MA).

Freeze fracture deep etching electron microscopy. Freeze fracture electron microscopy was achieved as described previously(62). Briefly, cultured BMECs were rapidly frozen by abrupt application of the sample against a liquid helium-cooled copper block with a Cryopress freezing machine. Samples were then moved to a liquid nitrogen-cooled Balzers 400 vacuum evaporator, fractured, and etched at -104°C for 2.5 min. For immuno-freeze fracture EM, etched samples were first stained with primary and gold-conjugated secondary antibodies on carbon-coated glass. These

472 samples were then rotary replicated with platinum (~2 nm), deposited from a 20° angle above the
 473 horizontal plane, followed by an immediate, stabilization film of pure carbon (~10 nm) deposited from
 474 an 85° angle. Replicas were floated onto bleach and transferred through multiple rinses of dH₂O before
 475 placing on formvar-coated EM grids. Electron micrographs were obtained using a JEM1400
 476 transmission microscope (JEOL) at 80 KV, equipped with an AMT XR111 4k digital camera.

477

478 **Statistical analysis.** Statistical analysis was performed using GraphPad Prism 7 software. A
 479 probability value of $p < 0.05$ was considered statistically significant. Statistical values are indicated as *,
 480 $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

481 **Acknowledgements**

482 This work was supported by Defense Threat Reduction Agency (DTRA) grants HDTRA1-15-1-0032
 483 (R.B.K.) and HDTRA1-15-1-0047 (W.B.K.) and NIH grants U19 AI083019, R01 NS052632, and R01
 484 AI101400, (R.S.K.). We have no conflicting interests to declare.

485 Correspondence and requests for materials should be addressed to R.S.K.

References

1. Charles PC, Walters E, Margolis F, Johnston RE. 1995. Mechanism of neuroinvasion of Venezuelan equine encephalitis virus in the mouse. *Virology* 208:662–671.
2. Phillips AT, Rico AB, Stauff CB, Hammond SL, Aboellail TA, Tjalkens RB, Olson KE. 2016. Entry Sites of Venezuelan and Western Equine Encephalitis Viruses in the Mouse Central Nervous System following Peripheral Infection. *J Virol* 90:5785–5796.
3. Fros JJ, Pijlman GP. 2016. Alphavirus Infection: Host Cell Shut-Off and Inhibition of Antiviral Responses. *Viruses* 8.
4. Bhalla N, Sun C, Metthew Lam LK, Gardner CL, Ryman KD, Klimstra WB. 2016. Host translation shutoff mediated by non-structural protein 2 is a critical factor in the antiviral state resistance of Venezuelan equine encephalitis virus. *Virology* 496:147–165.
5. Liebner S, Dijkhuizen RM, Reiss Y, Plate KH, Agalliu D, Constantin G. 2018. Functional morphology of the blood-brain barrier in health and disease. *Acta Neuropathol (Berl)* 135:311–336.
6. Dando SJ, Mackay-Sim A, Norton R, Currie BJ, John JAS, Ekberg JAK, Batzloff M, Ulett GC, Beacham IR. 2014. Pathogens Penetrating the Central Nervous System: Infection Pathways and the Cellular and Molecular Mechanisms of Invasion. *Clin Microbiol Rev* 27:691–726.
7. Lachenmaier SM, Deli MA, Meissner M, Liesenfeld O. 2011. Intracellular transport of *Toxoplasma gondii* through the blood-brain barrier. *J Neuroimmunol* 232:119–130.

- 505 8. Zhao Y-L, Song J-N, Zhang M. 2014. Role of caveolin-1 in the biology of the blood-brain barrier.
506 Rev Neurosci 25:247–254.
- 507 9. Andreone BJ, Chow BW, Tata A, Lacoste B, Ben-Zvi A, Bullock K, Deik AA, Ginty DD, Clish CB,
508 Gu C. 2017. Blood-Brain Barrier Permeability Is Regulated by Lipid Transport-Dependent
509 Suppression of Caveolae-Mediated Transcytosis. Neuron 94:581-594.e5.
- 510 10. Preston JE, Joan Abbott N, Begley DJ. 2014. Transcytosis of macromolecules at the blood-brain
511 barrier. Adv Pharmacol San Diego Calif 71:147–163.
- 512 11. Coyne CB, Bozym R, Morosky SA, Hanna SL, Mukherjee A, Tudor M, Kim KS, Cherry S. 2011.
513 Comparative RNAi Screening Reveals Host Factors Involved in Enterovirus Infection of Polarized
514 Endothelial Monolayers. Cell Host Microbe 9:70–82.
- 515 12. Van den Broeke C, Jacob T, Favoreel HW. 2014. Rho'ing in and out of cells. Small GTPases 5.
- 516 13. Persidsky Y, Heilman D, Haorah J, Zelivyanskaya M, Persidsky R, Weber GA, Shimokawa H,
517 Kaibuchi K, Ikezu T. 2006. Rho-mediated regulation of tight junctions during monocyte migration
518 across the blood-brain barrier in HIV-1 encephalitis (HIVE). Blood 107:4770–4780.
- 519 14. Afonso PV, Ozden S, Cumont M-C, Seilhean D, Cartier L, Rezaie P, Mason S, Lambert S, Huerre
520 M, Gessain A, Couraud P-O, Pique C, Ceccaldi P-E, Romero IA. 2008. Alteration of Blood–Brain
521 Barrier Integrity by Retroviral Infection. PLOS Pathog 4:e1000205.
- 522 15. Daniels BP, Holman DW, Cruz-Orengo L, Jujjavarapu H, Durrant DM, Klein RS. 2014. Viral
523 pathogen-associated molecular patterns regulate blood-brain barrier integrity via competing innate
524 cytokine signals. mBio 5:e01476-01414.

- 525 16. Miner JJ, Daniels BP, Shrestha B, Proenca-Modena JL, Lew ED, Lazear HM, Gorman MJ, Lemke
526 G, Klein RS, Diamond MS. 2015. The TAM receptor Mertk protects against neuroinvasive viral
527 infection by maintaining blood-brain barrier integrity. *Nat Med* 21:1464–1472.
- 528 17. Vogel P, Kell WM, Fritz DL, Parker MD, Schoepp RJ. 2005. Early events in the pathogenesis of
529 eastern equine encephalitis virus in mice. *Am J Pathol* 166:159–171.
- 530 18. Phillips AT, Rico AB, Stauff CB, Hammond SL, Aboellail TA, Tjalkens RB, Olson KE. 2016. Entry
531 Sites of Venezuelan and Western Equine Encephalitis Viruses in the Mouse Central Nervous
532 System following Peripheral Infection. *J Virol* 90:5785–5796.
- 533 19. Ronca SE, Dineley KT, Paessler S. 2016. Neurological Sequelae Resulting from Encephalitic
534 Alphavirus Infection. *Front Microbiol* 7.
- 535 20. Zacks MA, Paessler S. 2010. Encephalitic alphaviruses. *Vet Microbiol* 140:281–286.
- 536 21. Schäfer A, Brooke CB, Whitmore AC, Johnston RE. 2011. The Role of the Blood-Brain Barrier
537 during Venezuelan Equine Encephalitis Virus Infection. *J Virol* 85:10682–10690.
- 538 22. Wang E, Barrera R, Boshell J, Ferro C, Freier JE, Navarro JC, Salas R, Vasquez C, Weaver SC.
539 1999. Genetic and phenotypic changes accompanying the emergence of epizootic subtype IC
540 Venezuelan equine encephalitis viruses from an enzootic subtype ID progenitor. *J Virol* 73:4266–
541 4271.
- 542 23. Mossel EC, Ledermann JP, Phillips AT, Borland EM, Powers AM, Olson KE. 2013. Molecular
543 Determinants of Mouse Neurovirulence and Mosquito Infection for Western Equine Encephalitis
544 Virus. *PLOS ONE* 8:e60427.

- 545 24. Gardner CL, Burke CW, Tesfay MZ, Glass PJ, Klimstra WB, Ryman KD. 2008. Eastern and
546 Venezuelan equine encephalitis viruses differ in their ability to infect dendritic cells and
547 macrophages: impact of altered cell tropism on pathogenesis. *J Virol* 82:10634–10646.
- 548 25. Zhang R, Hryc CF, Cong Y, Liu X, Jakana J, Gorchakov R, Baker ML, Weaver SC, Chiu W. 2011.
549 4.4 Å cryo-EM structure of an enveloped alphavirus Venezuelan equine encephalitis virus. *EMBO*
550 *J* 30:3854–3863.
- 551 26. Kovtun O, Tillu VA, Ariotti N, Parton RG, Collins BM. 2015. Cavin family proteins and the
552 assembly of caveolae. *J Cell Sci* 128:1269–1278.
- 553 27. Predescu SA, Predescu DN, Malik AB. 2007. Molecular determinants of endothelial transcytosis
554 and their role in endothelial permeability. *Am J Physiol Lung Cell Mol Physiol* 293:L823-842.
- 555 28. Echarri A, Pozo MAD. 2015. Caveolae – mechanosensitive membrane invaginations linked to
556 actin filaments. *J Cell Sci* 128:2747–2758.
- 557 29. Komarova YA, Kruse K, Mehta D, Malik AB. 2017. Protein Interactions at Endothelial Junctions
558 and Signaling Mechanisms Regulating Endothelial Permeability. *Circ Res* 120:179–206.
- 559 30. Honnold SP, Mossel EC, Bakken RR, Lind CM, Cohen JW, Eccleston LT, Spurgers KB, Erwin-
560 Cohen R, Glass PJ, Maheshwari RK. 2015. Eastern equine encephalitis virus in mice II:
561 pathogenesis is dependent on route of exposure. *Virol J* 12:154.
- 562 31. Cain MD, Salimi H, Gong Y, Yang L, Hamilton SL, Heffernan JR, Hou J, Miller MJ, Klein RS. 2017.
563 Virus entry and replication in the brain precedes blood-brain barrier disruption during intranasal
564 alphavirus infection. *J Neuroimmunol* 308:118–130.

- 565 32. Sharma A, Bhomia M, Honnold SP, Maheshwari RK. 2011. Role of adhesion molecules and
566 inflammation in Venezuelan equine encephalitis virus infected mouse brain. Virol J 8:197.
- 567 33. Erbar S, Maisner A. 2010. Nipah virus infection and glycoprotein targeting in endothelial cells.
568 Virol J 7:305.
- 569 34. Soilu-Hänninen M, Erälinna JP, Hukkanen V, Røyttä M, Salmi AA, Salonen R. 1994. Semliki
570 Forest virus infects mouse brain endothelial cells and causes blood-brain barrier damage. J Virol
571 68:6291–6298.
- 572 35. Chhatbar C, Detje CN, Grabski E, Borst K, Spanier J, Ghita L, Elliott DA, Jordão MJC, Mueller N,
573 Sutton J, Prajeeth CK, Gudi V, Klein MA, Prinz M, Bradke F, Stangel M, Kalinke U. 2018. Type I
574 Interferon Receptor Signaling of Neurons and Astrocytes Regulates Microglia Activation during
575 Viral Encephalitis. Cell Rep 25:118-129.e4.
- 576 36. Nair S, Poddar S, Shimak RM, Diamond MS. 2017. Interferon Regulatory Factor 1 Protects
577 against Chikungunya Virus-Induced Immunopathology by Restricting Infection in Muscle Cells. J
578 Virol 91:e01419-17.
- 579 37. Gardner CL, Yin J, Burke CW, Klimstra WB, Ryman KD. 2009. Type I interferon induction is
580 correlated with attenuation of a South American eastern equine encephalitis virus strain in mice.
581 Virology 390:338–347.
- 582 38. Chanthick C, Suttiheptumrong A, Rawarak N, Pattanakitsakul S. 2018. Transcytosis Involvement
583 in Transport System and Endothelial Permeability of Vascular Leakage during Dengue Virus
584 Infection. Viruses 10:69.

- 585 39. Chanthick C, Kanlaya R, Kiatbumrung R, Pattanakitsakul S, Thongboonkerd V. 2016. Caveolae-
586 mediated albumin transcytosis is enhanced in dengue-infected human endothelial cells: A model
587 of vascular leakage in dengue hemorrhagic fever. *Sci Rep* 6:31855.
- 588 40. Brown RS, Wan JJ, Kielian M. 2018. The Alphavirus Exit Pathway: What We Know and What We
589 Wish We Knew. *Viruses* 10.
- 590 41. Laakkonen P, Auvinen P, Kujala P, Kääriäinen L. 1998. Alphavirus replicase protein NSP1
591 induces filopodia and rearrangement of actin filaments. *J Virol* 72:10265–10269.
- 592 42. Ahola T, Kujala P, Tuittila M, Blom T, Laakkonen P, Hinkkanen A, Auvinen P. 2000. Effects of
593 Palmitoylation of Replicase Protein nsP1 on Alphavirus Infection. *J Virol* 74:6725–6733.
- 594 43. Knowland D, Arac A, Sekiguchi KJ, Hsu M, Lutz SE, Perrino J, Steinberg GK, Barres BA,
595 Nimmerjahn A, Agalliu D. 2014. Stepwise recruitment of transcellular and paracellular pathways
596 underlies blood-brain barrier breakdown in stroke. *Neuron* 82:603–617.
- 597 44. Castejón OJ. 1980. Electron microscopic study of capillary wall in human cerebral edema. *J*
598 *Neuropathol Exp Neurol* 39:296–328.
- 599 45. Claudio L, Raine CS, Brosnan CF. 1995. Evidence of persistent blood-brain barrier abnormalities
600 in chronic-progressive multiple sclerosis. *Acta Neuropathol (Berl)* 90:228–238.
- 601 46. Long M, Huang S-H, Wu C-H, Shackleford GM, Jong A. 2012. Lipid raft/caveolae signaling is
602 required for *Cryptococcus neoformans* invasion into human brain microvascular endothelial cells.
603 *J Biomed Sci* 19:19.

- 604 47. Kiss AL, Botos E. 2009. Endocytosis via caveolae: alternative pathway with distinct cellular
605 compartments to avoid lysosomal degradation? J Cell Mol Med 13:1228–1237.
- 606 48. Tugizov SM, Herrera R, Palefsky JM. 2013. Epstein-Barr Virus Transcytosis through Polarized
607 Oral Epithelial Cells. J Virol 87:8179–8194.
- 608 49. Kinlock BL, Wang Y, Turner TM, Wang C, Liu B. 2014. Transcytosis of HIV-1 through vaginal
609 epithelial cells is dependent on trafficking to the endocytic recycling pathway. PloS One 9:e96760.
- 610 50. Papa MP, Meuren LM, Coelho SVA, Lucas CG de O, Mustafá YM, Lemos Matassoli F, Silveira
611 PP, Frost PS, Pezzuto P, Ribeiro MR, Tanuri A, Nogueira ML, Campanati L, Bozza MT, Paula
612 Neto HA, Pimentel-Coelho PM, Figueiredo CP, de Aguiar RS, de Arruda LB. 2017. Zika Virus
613 Infects, Activates, and Crosses Brain Microvascular Endothelial Cells, without Barrier Disruption.
614 Front Microbiol 8:2557.
- 615 51. Chaudhary N, Gomez GA, Howes MT, Lo HP, McMahon K-A, Rae JA, Schieber NL, Hill MM,
616 Gaus K, Yap AS, Parton RG. 2014. Endocytic crosstalk: cavins, caveolins, and caveolae regulate
617 clathrin-independent endocytosis. PLoS Biol 12:e1001832.
- 618 52. Favoreel HW, Enquist LW, Feierbach B. 2007. Actin and Rho GTPases in herpesvirus biology.
619 Trends Microbiol 15:426–433.
- 620 53. Locker JK, Kuehn A, Schleich S, Rutter G, Hohenberg H, Wepf R, Griffiths G. 2000. Entry of the
621 Two Infectious Forms of Vaccinia Virus at the Plasma Membrane Is Signaling-Dependent for the
622 IMV but Not the EEV. Mol Biol Cell 11:2497–2511.

- 623 54. Schowalter RM, Wurth MA, Aguilar HC, Lee B, Moncman CL, McCann RO, Dutch RE. 2006. Rho
624 GTPase activity modulates paramyxovirus fusion protein-mediated cell–cell fusion. *Virology*
625 350:323–334.
- 626 55. Drewry LL, Jones NG, Wang Q, Onken MD, Miller MJ, Sibley LD. 2019. The secreted kinase
627 ROP17 promotes *Toxoplasma gondii* dissemination by hijacking monocyte tissue migration. *Nat*
628 *Microbiol.*
- 629 56. Agarwal V, Hammerschmidt S. 2009. Cdc42 and the Phosphatidylinositol 3-Kinase-Akt Pathway
630 Are Essential for PspC-mediated Internalization of Pneumococci by Respiratory Epithelial Cells. *J*
631 *Biol Chem* 284:19427–19436.
- 632 57. Blakely PK, Delekta PC, Miller DJ, Irani DN. 2015. Manipulation of host factors optimizes the
633 pathogenesis of western equine encephalitis virus infections in mice for antiviral drug
634 development. *J Neurovirol* 21:43–55.
- 635 58. Boroujerdi A, Tigges U, Welser-Alves JV, Milner R. 2014. Isolation and culture of primary pericytes
636 from mouse brain. *Methods Mol Biol Clifton NJ* 1135:383–392.
- 637 59. Weksler BB, Subileau EA, Perrière N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H,
638 Nicotra A, Bourdoulous S, Turowski P, Male DK, Roux F, Greenwood J, Romero IA, Couraud PO.
639 2005. Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J*
640 *Off Publ Fed Am Soc Exp Biol* 19:1872–1874.

- 641 60. Sun C, Gardner CL, Watson AM, Ryman KD, Klimstra WB. 2014. Stable, high-level expression of
642 reporter proteins from improved alphavirus expression vectors to track replication and
643 dissemination during encephalitic and arthritogenic disease. *J Virol* 88:2035–2046.
- 644 61. Cain MD, Salimi H, Gong Y, Yang L, Hamilton SL, Heffernan JR, Hou J, Miller MJ, Klein RS. 2017.
645 Virus entry and replication in the brain precedes blood-brain barrier disruption during intranasal
646 alphavirus infection. *J Neuroimmunol* 308:118–130.
- 647 62. Heuser JE, Kirschner MW. 1980. Filament organization revealed in platinum replicas of freeze-
648 dried cytoskeletons. *J Cell Biol* 86:212–234.

649

FIG. 1

650

651 **FIG 1** Alphavirus infection of the CNS occurs prior to BBB disruption. (A and B) Viral burdens in brain
 652 tissues of C57BL/6 mice following f.p. infection with either VEEV (10 pfu) or WEEV (1000 pfu),

determined by plaque assay. Dashed lines indicate the limit of detection of the assay. Viral burdens were measured at 1 dpi (VEEV) and 3 dpi (WEE). (C and D) BBB permeability was determined at indicated dpi by measuring sodium fluorescein in CNS tissues following i.p. administration. Data presented as mean viral titer or fluorescence $\pm$ SEM for N=5-10 mice/group. *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$, via 1-way ANOVA. (E) A schematic figure of an *in vitro* BBB model. (F and G) Neither exposure of BMECs nor astrocytes (ACs) to VEEV and WEEV viruses had any effects on barrier integrity. Addition of TNF- α (100 ng/ml) significantly reduced TEER over time. Experiment was performed twice, each with 6 replicates. Error bars indicate mean $\pm$ SEM. Statistically significant differences were determined via 2-way ANOVA followed by Dunnett's multiple comparison test.

FIG 2.

FIG 2 Hematogenous route of alphavirus neuroinvasion is not exclusive to CVOs. (A and B) Low magnification of a sagittal section of VEEV-eGFP- (A) and WEEV-eGFP (B) infected mouse brain showing multiple entry sites for CNS entry. Brain tissues from VEEV-eGFP (C) and WEEV-eGFP (D) infected mice were stained for makers of astrocytes (S100-β, Magenta), BMECs (CD31, Red), pericytes (PDGFR-β, Red) and neurons (NeuN, Magenta). Insets are enlarged in lower left of each panel. Single channels relate to the enlarged insets. Nuclei were counterstained with DAPI (blue). Magnification 40x, Scale bars: 20 μm.

FIG. 3

FIG 3 Type I IFNs differentially restrict alphavirus replication within the NVU. IHC staining of cortical brain regions of WT (A and C) and *Ifnar*^{-/-} (B and D) mice following f.p. infection with either VEEV-eGFP (A and B) or WEEV-eGFP (C and D). Cell markers: astrocytes (S100-β), BMECs (CD31), pericytes (PDGFR-β) and neurons (NeuN). Nuclei counterstained with DAPI (blue). (E and F) IHC staining of

680 cortical brain regions of WT and *Ifnar*^{-/-} mice following i.v. infection with NR-VEEV-eGFP. Magnification
681 40x. Scale bars: 20 μm.

682

FIG. 4

FIG 4 Detection of alphavirus interaction and entry at the BBB. (A) TEM analysis of brain tissues from VEEV infected mice at 1 dpi, identified virus like particles attached to luminal surface of microvessels. (B) Viral E2 glycoproteins were detected within cortical BMECs of VEEV-eGFP infected mice (3 dpi) by immune-EM using 8 nm immunogold particles. Scale bars=100 nm. (C and D) Immunostaining of brain tissues collected from NR VEEV-eGFP infected WT mice at 1 day following i.v. infection. Cell markers: BMECs (CD31), and neurons (NeuN). Nuclei counterstained with DAPI (blue). Magnification 40x. Scale bars: 20 μm.

694 compared to vehicle. (D) Quantitation of cell surface-associated caveolae in BMECs after virus
 695 exposure. Statistical differences were analyzed by 1-way ANOVA followed by Dunnett's multiple
 696 comparison test. ***, $P < 0.001$. (E and F) TEM analysis of brain tissues collected from naïve and
 697 VEEV-infected mice at 3 dpi. (G) Quantitation of caveolae-like structures at the cell surface and inside
 698 cytoplasm of brain endothelial cells of naïve vs infected animals. Three tissue blocks were harvested
 699 from cortical brain region of each mouse (N=3 in each group), and 30 images were obtained per block.
 700 After manual counting, the mean vesicular density per length or volume of cytoplasm was calculated
 701 using ImageJ (NIH). Statistical differences were analyzed using t-test. (H) TEM analysis demonstrating
 702 membrane ruffling in BMECs of infected mice relative to control animals.

FIG. 6

FIG 6 Alphavirus crosses BMECs via caveolae-mediated transcytosis. (A) A schematic figure demonstrating different steps of a transcytosis assay. (B) Addition of a non-replicative strain of VEEV-eGFP to hCMEC/D3 on the top chamber resulted in GFP signals in human astrocytes in bottom chamber. (C-E) TEM analysis identified virus like particles (depicted by arrows) within multi-vesicular bodies (D) and exotic endosomes (E) in BMECs after exposure to VEEV for 30 min. (F-I) VEEV and WEEV traverse across monolayers of BMEC (F and H) and hCMEC/D3 (G and I) via caveolae-mediated transcytosis. Data are presented as mean % of transcytosis relative to untreated cells. Experiments were repeated 2-3 times each with 4-6 technical replicates. Error bars indicate standard error of the mean (SEM). Concentration of inhibitors: Amiloride hydrochloride hydrate (50 μ m), Cholesterol oxidase (CHOD, 2 U/ml), filipin (1 μ g/ml), nystatin (12 μ g/ml), chlorpromazine (CPZ, 10 μ g/ml). (J) Quantitation of VEEV transcytosis across WT and *Cav-1*^{-/-} BMECs. (K) Immunogold labeling of virally infected BMECs revealed VEEV (8 nm immunogold particles; orange arrow) in colocalization with caveoline-1 (16 nm immunogold particles; white arrow). (L) VEEV transcytosis was compared between brain and lung ECs. (M and N) VEEV transcytosis across BMECs in the absence and presence of Rho kinase GTPase (H-1152P) and Rac1 (Z62954982), as well as type I IFNs (100 pg/ml). Results from transcytosis assays were analyzed by 1-way ANOVA (F-I, M and N), and t-test (J and L). *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$.

FIG. 7

FIG 7 Cav-1^{-/-} mice displayed reduced viral titers in the brain as compared to WT animals. (A and B) Viral burdens in peripheral and brain tissues of WT vs Cav-1 KO mice after f.p. infection with VEEV (10 pfu) and WEEV (1000 pfu), determined by plaque assay. Error bars indicate standard error of the mean (SEM). Shown is the combined data from 4 independent experiments each with 4-6 animals. Results were analyzed by unpaired t-test. *, P < 0.05; **, P < 0.01.

Supplementary Figures:

FIG. S1

FIG S1 Alphavirus enters the CNS in the presence of an intact BBB. (A and B) Viral burdens in brain tissues of C57BL/6 mice following f.p. infection with either VEEV (10 PFU) or WEEV (1000 pfu) were determined via plaque assay. Dashed lines indicates detection limit of the assay. (C and D) BBB permeability was evaluated at indicated dpi by measuring sodium fluorescein in CNS tissues following i.p. injection. Results are the combined data of two independent experiments. Data presented as mean viral titer or fluorescence $\pm$ SEM for N=5-10 mice/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, via 1-way ANOVA.

FIG. S2

FIG S2 Cells of the neurovascular unit are permissive to alphavirus infection *in vitro*. Replication kinetic of VEEV and WEEV in murine (A-C, and E-G) and human cells (D and H) of the NVU. Multi-step growth curves were generated using Graphpad Prism 7. Shown is a representative data from three independent experiments each in duplicates.

FIG. S3

FIG S3 Survival phenotype of WT and *Ifnar*^{-/-} mice following alphavirus infection. (A and B) WT and *Ifnar*^{-/-} mice were infected with VEEV (10 pfu) and WEEV (1000 pfu) via foot-pad injection. Infected animals were monitored for survival and weight loss for 20 days. Shown is the combined data from 2 independent experiments with 3-4 animals each time. (C) Viral titers in the brain of WT versus *Ifnar*^{-/-} following infection with VEEV-eGFP (100 pfu) at indicated time-point. (D and E) IHC examining of brain tissues collected from infected WT (D) and *Ifnar*^{-/-} (E) mice at 1 dpi. Cell markers: astrocytes (S100-β), BMECs (CD31), pericytes (PDGFR-β) and neurons (NeuN). Nuclei counterstained with DAPI (blue). Magnification 40x. Scale bar: 20 μm.

FIG. S4

FIG S4 IFN-mediated restriction of alphavirus within NVU is uniform across the brain. IHC staining of brain tissues from WT (3dpi; A and C) and *Ifnar*^{-/-} (2 dpi; B and D) mice infected with either 100 pfu of VEEV-eGFP (A and B) or 1000 pfu of WEEV-eGFP via f.p. injection (C and D). Cerebellum sections were stained for markers of astrocytes (S100-β), BMECs (CD31), pericytes (PDGFR-β) and neurons

773 (NeuN or Calmodulin). Nuclei counterstained with DAPI (blue). Magnification 40x. Scale bars: 20 μ m.
 774 (E) Infection of cerebral BMECs and pericytes in *Ifnar*^{-/-} mice following *i.v.* infection with a non-
 775 replicative (NR) replicon of VEEV-eGFP. (F) Serum titers of VEEV in WT versus IFNAR^{-/-} mice following
 776 f.p. infection.

777

FIG. S5

FIG S5 Replication kinetics of VEEV in BMECs. (A) BMECs were infected with VEEV at a MOI of 2 for 30 min. After extensive washes, virus production was assayed in the culture medium at indicated time-points using plaque assay. (B and C) Effects of inhibitors of endocytic pathways on barrier integrity, which was determined by measuring TEER before and after addition of inhibitors to BMECs on the top chamber of a transwell plate. (D and E) Effects of endocytosis inhibitors on virus infectivity. Viruses were incubated with inhibitors or vehicle for 1 hr, followed by assessment of virus infectivity using plaque assay in BHK-21 (VEEV) or Vero (WEEV) cell lines. Drug concentrations: Amiloride hydrochloride hydrate (50 μM), Cholesterol oxidase (CHOD, 2 U/ml), filipin (1 $\mu\text{g/ml}$), nystatin (12 $\mu\text{g/ml}$), chlorpromazine (CPZ, 10 $\mu\text{g/ml}$), H-1152P (50 nm, 100 nm), Z62954982 (100 μM), IFN- α (100 pg/ml), IFN- β (100 pg/ml).

FIG. S6

FIG S6 Difficinecy in *Cav-1* does not affect alphavirus replication efficiency within the CNS. (A and B) Viral titers in peripheral and brain tissues of WT vs *Cav-1*^{-/-} mice at 1dpi following i.c. infection with VEEV (10 PFU) and WEEV (100 pfu). Error bars indicate standard error of the mean (SEM).