West Nile virus-induced disruption of the blood–brain barrier in mice is characterized by the degradation of the junctional complex proteins and increase in multiple matrix metalloproteinases

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West Nile virus (WNV) encephalitis is characterized by neuroinflammation, neuronal loss and blood–brain barrier (BBB) disruption. However, the mechanisms associated with the BBB disruption are unclear. Complex interactions between the tight junction proteins (TJP) and the adherens junction proteins (AJP) of the brain microvascular endothelial cells are responsible for maintaining the BBB integrity. Herein, we characterized the relationship between the BBB disruption and expression kinetics of key TJP, AJP and matrix metalloproteinases (MMPs) in the mice brain. A dramatic increase in the BBB permeability and extravasation of IgG was observed at later time points of the central nervous system (CNS) infection and did not precede virus–CNS entry. WNV-infected mice exhibited significant reduction in the protein levels of the TJP ZO-1, claudin-1, occludin and JAM-A, and AJP β-catenin and vascular endothelial cadherin, which correlated with increased levels of MMP-1, -3 and -9 and infiltrated leukocytes in the brain.

Further, intracranial inoculation of WNV also demonstrated increased extravasation of IgG in the brain, suggesting the role of virus replication in the CNS in BBB disruption. These data suggest that altered expression of junction proteins is a pathological event associated with WNV infection and may explain the molecular basis of BBB disruption. We propose that WNV initially enters CNS without altering the BBB integrity and later virus replication in the brain initiates BBB disruption, allowing enhanced infiltration of immune cells and contribute to virus neuroinvasion via the ‘Trojan-horse’ route. These data further implicate roles of multiple MMPs in the BBB disruption and strategies to interrupt this process may influence the WNV disease outcome.

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne neurotropic flavivirus responsible for the largest outbreak of viral meningoencephalitis in the Western Hemisphere since its appearance in New York in 1999. A subset of WNV patients with febrile illness progress to encephalitis (Davis et al., 2006; Gubler, 2007) and neurological disease syndrome is observed in approximately 30% of confirmed WNV cases with a higher frequency in the elderly and immunocompromised (Campbell et al., 2002; Hayes & Gubler, 2006). No specific therapy for WNV is currently approved for use in humans. In the mouse model, WNV-associated neurological disease is characterized by disruption of the blood–brain barrier (BBB), increased infiltration of immune cells into the central nervous system (CNS), production of inflammatory cytokines, microglia activation and eventual loss of neurons (Glass et al., 2005; Klein et al., 2005; Samuel & Diamond, 2006). WNV entry into the CNS represents an important event in the clinical outcome of WNV. The proposed routes of entry are by crossing the BBB and by retrograde axonal transport via peripheral nervous system (Samuel et al., 2007). Since high viraemia correlates with early WNV entry into the CNS, haematogenous route via crossing the BBB is suggested to be one of the major routes by which WNV enters the CNS (King et al., 2007) although the associated mechanisms are yet unclear. We previously demonstrated in vitro that WNV can infect and efficiently replicate in human brain microvascular endothelial cells, which line the BBB, and can be one of the routes of virus entry into the CNS without affecting the BBB integrity (Verma et al., 2009).

The BBB, which is the interface between circulating blood and the CNS, is composed of specialized microvascular
endothelial cells and perivascular astrocytes separated by the basement membrane (Abbott, 2005; Persidsky et al., 2006). The BBB-endothelial cells are unique because of the presence of specific junctional complex proteins that seal the intracellular gaps between adjacent endothelial cells, resulting in the formation of a selective barrier that regulates the entrance of circulating leukocytes and pathogens into the brain (Persidsky et al., 2006). The TJP complex comprises transmembrane proteins, including occludin and claudins that interact with cytosolic adaptor proteins, the zona occludins (ZO), which connect to apical actin filaments forming a tight seal between adjacent cells. The key proteins in the AJ complex are the transmembrane vascular endothelial cadherin (VE-cadherin) and cytosolic catenins (Bazzoni et al., 2000; Zlokovic, 2008). This continuous AJ complex holds neighbouring cells together and assists in stabilizing the interactions between TJP, thereby supporting the integrity of the BBB and regulating paracellular permeability (Bazzoni et al., 2000). Junctional complex proteins are highly sensitive to the microenvironment and respond to inflammatory molecules, resulting in alterations in their subcellular distribution and/or dissociation of the transmembrane-adaptor protein complexes.

Several neurotropic pathogens have evolved strategies to cross the BBB to gain access to the CNS, including direct infection of BBB cells, paracellular entry through the compromised BBB and transmigration within infected leukocytes via the Trojan-horse mechanism. Increased permeability of the BBB is a pathological hallmark in several neurological disorders such as multiple sclerosis, bacterial meningitis (Huber et al., 2001) and neurotropic virus infections including human immunodeficiency virus (HIV) (Dallasta et al., 1999), measles virus (Cosby & Brankin, 1995), mouse adenovirus (Gralinski et al., 2009) and arthropod-borne viruses such as Japanese encephalitis virus (JEV) (Mishra et al., 2009), WNV (Morrey et al., 2000; Wang et al., 2004) and Venezuelan equine encephalitis virus (VEEV) (Schäfer et al., 2011). BBB disruption is associated with the degradation of specific junction proteins, which contributes to virus entry (Afonso et al., 2007; Luabeya et al., 2000) and enhanced transmigration of activated immune cells into the brain (Boven et al., 2000). These events correlate with simultaneous production of matrix-degrading metalloproteinases (MMPs), a large family of endopeptidases in the region of injury (Rosenberg, 1995). In CNS infections, MMPs are thought to play a major role in promoting destructive neuroinflammatory processes including BBB disruption via TJP degradation, oedema formation and disintegration of the neurovascular unit (Liu & Rosenberg, 2005; Rosenberg, 2002).

Previous studies have demonstrated that BBB integrity is compromised in WNV-infected mice; however, the underlying mechanisms are not well defined. It is hypothesized that the cascade of the production of proinflammatory mediators such as tumour necrosis factor alpha, MMP-9 and macrophage migration inhibitory factor may alter the BBB integrity (Arjona et al., 2007; Wang et al., 2004, 2008a). Our previous in vitro studies provide evidence for the role of WNV-induced MMPs in human astrocytes in compromising the integrity of the in vitro BBB model (Verma et al., 2010). Additionally, Wang et al. (2008a) demonstrated that MMP-9−/− mice were resistant to WNV infection and markers of BBB disruption such as IgG extravasation and Evans blue leakage into the brain were markedly reduced in the MMP9−/− mice compared with controls. Although these studies though collectively suggest the pathogenic role of BBB disruption and MMPs in WNV neuropathogenesis, the precise mechanism, specifically the effect of WNV on the expression kinetics of key BBB junction proteins has not been delineated in vivo. Therefore, in this study we characterized the expression pattern of multiple TJP and AJP in the mouse brains and correlated it with the kinetics of BBB disruption, MMPs production and infiltration of leukocytes during the course of infection.

RESULTS

In vivo WNV-induced BBB disruption correlates with peak viral titres in the brain

To understand the specific in vivo mechanisms by which WNV mediates BBB disruption, we examined the kinetics of BBB disruption during the course of WNV infection by measuring the leakage of Evans blue dye and systemic IgG proteins into mice brains. Evans blue is a cationic dye that binds to the albumin present in the sera and forms a complex that cannot pass through the intact BBB. Extravasation of this complex into the brain is indicative of increased BBB permeability. Our results demonstrate that in the mock-infected animals the Evans blue dye perfused only into the peripheral tissues such as spleen and kidney but not into the brain (Fig. 1a) thereby suggesting an intact BBB. In WNV-infected mice, the BBB was observed to be intact at days 2 and 4 after infection, similar to the control brains. However, detection of leakage of the dye was first observed at day 6 after infection, which became more prominent at day 8 after infection (Fig. 1a). In those mice that survived WNV infection, no leakage of Evans blue dye was observed in the brain at day 21 after infection, suggesting restoration of the BBB integrity (data not shown). Similarly, Fig. 1(b) indicates that IgG (heavy and light chains) levels in the perfused brains increased significantly in the brains at day 8 after infection in comparison to the mock-infected brains, further validating that compromised BBB leads to increased transmigration of systemic proteins in the brain. The WNV titres in the sera displayed peak viraemia in the sera at days 3 and 4 after infection (Fig. 1c). In the brains, while WNV was not detected at day 4 after infection, the titres reached 10⁴ p.f.u. µg⁻¹ RNA at day 6 and
further increased at day 8 after infection (Fig. 1d). Overall, the kinetics of BBB disruption demonstrating compromised integrity at day 8 after infection correlated positively with the peak virus titres in the brain.

**BBB disruption is accompanied by decreased protein levels of TJP and AJP**

To further understand the relationship between BBB disruption and the levels of junctional proteins, we analysed the mRNA and protein levels of multiple TJP and AJP in the infected-brain tissue. As determined by quantitative real-time PCR (qRT-PCR), the mRNA levels of the TJP claudin-1, occludin, ZO-1 and JAM-A revealed no significant change at any time points following WNV infection (Fig. 2a). However, the protein levels of these TJP, as determined by Western blot analysis, were significantly altered in the brains of infected mice. Except for JAM-A, the protein levels of claudin-1, occludin and ZO-1 did not change significantly in the brain at day 4 after infection; however, by day 6 after infection, the decrease in these TJP was approximately 20–50% as compared with controls (Fig. 2b). This decrease became even more pronounced at day 8 after infection. As seen in Fig. 2(c), decrease in the expression of claudin-1 and occludin was 70–80% (P < 0.05) and JAM-A was approximately 85% (P < 0.001) as compared with controls at day 8 after infection. Since in vivo AJP are also critical for maintaining the paracellular permeability across the BBB, the expression profile of β-catenin and VE-cadherin was also analysed. As depicted in Fig. 3(a), compared to controls no significant changes were observed in the mRNA expression of AJP, β-catenin and VE-cadherin in WNV-infected brains. Similar to the TJP, while WNV infection did not alter protein levels of these AJP at day 4 after infection, they decreased by approximately 40 and 60% at days 6 and 8 after infection, respectively (Fig. 3b and c).

Alterations in the expression of brain TJP and AJP were further validated by immunostaining of the brain sections harvested at day 8 after infection. We also examined the brain sections for the expression of glial fibrillary acidic protein (GFAP), a marker of astrocytes activation and von Willebrand Factor (vWF), a specific marker of microvascular endothelial cells. As seen in Fig. 4, increased immunostaining of GFAP was observed in infected brain and indicates activation of astrocytes, a well characterized feature of WNV-associated pathology. However, the fluorescence intensity of vWF staining was similar in both mock- and WNV-infected brain sections, suggesting similar capillary morphology. In brain sections from mock-infected mice, distinct immunostaining of TJP and AJP surrounding the capillaries was evident. However, the fluorescent signal of all the TJP and AJP examined decreased dramatically in WNV-infected brain sections. In contrast to linear and continuous staining pattern in control tissue, immunoreactivity of claudin-1 and ZO-1 in infected tissue resembled spot-like structures. Immunostaining of occludin, JAM-A and β-catenin in infected tissue also demonstrated either loss of immunoreactivity or areas of fragmented staining (Fig. 4). These results are consistent with the Western blot data (Fig. 3b) and collectively indicate that the severe loss of both TJP

![Fig. 1. WNV infection induces BBB disruption in vivo. Mice were inoculated with 100 p.f.u. of WNV via footpad, and sera and brain were harvested at different time points after infection. (a) Measurement of the BBB permeability using Evans blue dye at days 2, 4, 6 and 8 after infection. Mice were injected i.p. with 1 ml Evans blue dye (1 % w/v) and after 1 h were cardiac perfused with PBS. The dye diffused into the peripheral tissues and turned them blue. The extravasation of the dye was evident in the brains harvested at days 6 and 8 after infection. (b) Brain lysates were resolved by SDS-PAGE and Western blotting was conducted to detect heavy and light chains of IgG. (c) WNV titres in the serum were determined by plaque assay using Vero cells and were expressed as p.f.u. ml⁻¹ serum and (d) WNV copy numbers in the brain of WNV-infected mice were determined by qRT-PCR and were expressed as p.f.u. µg⁻¹ RNA. M, Mock infected.](http://vir.sgmjournals.org)
and AJP protein in the brain correlates with the BBB disruption (Fig. 1a).

We next assessed the correlation of BBB disruption with the infiltration of leukocytes in the brain. Haematoxylin and eosin stained sections demonstrated the presence of infiltrating leukocytes in the meninges of the brain at day 8 after infection (Fig. 5a). Further, while infiltrating leukocytes represented by CD11b+ cells (monocytes and neutrophils) in the brains did not increase at day 6 (data not shown), the increase was significant at day 8 after infection and correlated positively with BBB disruption (Fig. 5b).

Multiple MMPs are induced by WNV in vivo

To determine if the decreased TJP and AJP levels (Figs 2, 3, 4) could be explained through the actions of MMPs, we analysed the expression levels of multiple MMPs in the brain following WNV infection. A six- to eightfold increase in the mRNA transcripts of MMP-1 and -3 were observed in the brain at day 6 after infection, which further increased at day 8 after infection (Fig. 6a). While the mRNA expressions of MMP-9 did not increase at day 6 after infection, a modest twofold increase of MMP-9 was observed in the infected brain tissue at day 8 after infection (Fig. 6a). The expression of tissue inhibitor of metalloproteinases 2 (TIMP-2) and MMP-2 in the brain did not alter at any time points following WNV infection (data not shown).

Western blotting further demonstrated the increase of MMP-3 and -9 at days 6 and 8 after infection (Fig. 6b). Since MMPs are secretory proteins, their levels were also examined in the serum and brain using ELISA. Surprisingly, as compared to mock, MMP-3 levels did not change in the serum although a significant increase in MMP-9 levels was observed at day 3 after infection ($P<0.05$), which returned to the basal level by day 6 and remained low at day 8 after infection (Fig. 6c). However, in the brain, levels of MMP-3 and -9 increased significantly at day 8 as compared with controls, thus coinciding with the increase in their mRNA transcripts at the same time points (Fig. 6d). The positive correlation between the BBB disruption and increased MMPs in the brain and not in the serum, suggest that inflammatory processes within the CNS might be critical in TJP and AJP degradation.

WNV infection via intracranial route also induces BBB disruption

As we observed that the time points of BBB disruption coincided with high MMP levels and virus titres in the brain and not in the serum, we hypothesized that decreased TJP and AJP expression could be a result of increased virus replication-induced inflammation in the brain. To validate this hypothesis, we inoculated mice with similar infectious dose of WNV (100 p.f.u.) directly into the cranium and

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**Fig. 2.** BBB disruption is characterized by decrease in TJP protein. (a) qRT-PCR was conducted on RNA extracted from brains harvested from mice inoculated via footpad to determine mRNA fold-change of claudin-1, occludin, ZO-1 and JAM-A expression at days 2, 4, 6 and 8 after WNV infection. Data represents the means±SEM of at least five mice conducted in duplicate. (b) Total brain lysates were separated by SDS-PAGE, transferred onto PVDF membranes and immunoblotted with antibodies specific to claudin-1, occludin, ZO-1 and JAM-A. Equal loading was confirmed by reprobing with anti-β-actin antibody and the bands were detected using the Li-Cor Odyssey infrared method. (c) Quantitative analysis of Western blot results represented as a ratio between TJP and β-actin in control and infected brain tissue. Mean comparisons were based on data from at least four mice. *$P<0.05$ as compared to mock controls (M).
As compared to mock brains, the mRNA expression of β-catenin and VE-cadherin did not change in the brains at days 2, 4, 6 and 8 after infection via the footpad route as determined by qRT-PCR. Data represent the means ± SEM of at least five mice conducted in duplicate. (b) Protein expression of β-catenin and VE-cadherin as determined by Western blot analysis was significantly reduced in the brains at days 6 and 8 after infection. (c) Quantitative analysis of Western blot results. All values are relative to β-actin and represent at least four samples per time point. *P<0.05 as compared to mock controls (M).

**DISCUSSION**

Impaired function of the brain vasculature can contribute to the trafficking of pathogens and activated immune cells into the CNS causing death of neurons. Previous studies have demonstrated that BBB permeability is compromised in WNV-infected mice; however, the associated mechanisms have not been elucidated so far in vivo. In this report, we demonstrate that WNV-induced BBB disruption occurs gradually during the course of infection but does not precede WNV-CNS entry. Further, BBB disruption results in significant alterations in the expression of TJP and AJP, which correlate with peak WNV titres, increased production of MMPs and infiltrated leukocytes in the brain. In summary, these data suggest that initially, WNV enters into the CNS without disrupting the BBB, and initiates virus replication and inflammation, which leads to the opening of the BBB allowing unrestricted entry of virus and immune cells into the CNS.

**WNV infection disrupts the BBB**

Inflammation of the CNS accompanied by migration of activated immune cells into the CNS and increased BBB permeability are vital components of encephalitis-associated with neurotropic viruses including HIV, human T-cell lymphotropic virus (HTLV) and flaviviruses such as JEV, WNV and Murray Valley encephalitis virus (Afonso et al., 2007; Dallasta et al., 1999; King et al., 2007; Mishra et al., 2009). Previous studies demonstrating increased mortality by administration of lipopolysaccharide, which is known to enhance BBB permeability, in mice infected with non-neuroinvasive strain of WNV supports the fact that BBB disruption contributes to virus–CNS entry and disease outcome (Lustig et al., 1992). Our data using two well-established methods of BBB permeability assay, suggest that changes in BBB permeability develop slowly during the course of infection and the most dramatic decrease in its integrity is observed when virus infection is already established in the CNS. Though intrathecal antibody production can also contribute to the IgG in the brain, the presence of systemic proteins including IgG is an established indicator of compromised BBB integrity. The BBB disruption we observed concurs with previous reports wherein IgG extravasation was detected in the brain at day 7 after infection (Wang et al., 2008a). Further, Wang et al. (2004) also established the role of the BBB disruption in WNV pathogenesis; however, they observed BBB disruption as early as day 3 after infection, which may be because of either higher infectious dose (LD100) inoculated via intraperitoneal route or a different strain of the WNV. On the other hand, Morrey et al. (2008) did not report correlation between increased BBB permeability and lethality of WNV-infected rodents. These differences could be attributed to the virus dose used for inoculation (10^5 p.f.u.), which resulted in early mortality, starting at day 6 after infection (Morrey et al., 2008).

**Degradation of junction proteins is a pathological hallmark of WNV infection**

In this report, for the first time we demonstrate that WNV infection results in the degradation of multiple TJP in the
mouse brain. Since the mRNA levels of these TJP did not alter, it indicates that WNV does not affect the expression of these TJP at the transcriptional level. AJP form a continuous belt that serves to hold neighbouring cells together; hence their importance in maintaining the BBB integrity. Similar to TJP, significant reduction of both AJP β-catenin and VE-cadherin was only observed at the protein level in the WNV-infected mice brain. The degradation of TJP in compromised BBB is very well established in virus infections, but understanding of the roles of AJP in morphological changes of the BBB and associated pathogenesis is limited. Therefore, our results are important since they suggest that in addition to TJP, expression of AJP is also severely compromised in the event of WNV-associated BBB disruption. Based on our findings we postulate that the marked decrease in the junctional complex proteins may be the cause of altered BBB permeability observed in WNV-infected mice. Interestingly, recent studies have linked the decrease in the levels of VE-cadherin and ZO-1 in dengue virus-infected human endothelial cells with vascular leakage observed in dengue shock syndrome, suggesting that modulation of junction proteins may be a common phenomenon.
in flavivirus infection (Kanlaya et al., 2009). Correlation of breakdown of TJP with BBB disruption and leukocyte infiltration has been well established in infection with viruses such as HIV, HTLV and mouse adenovirus (Afonso et al., 2007; Gralinski et al., 2009; Kanmogne et al., 2007).

MMPs are widely implicated in modulating BBB integrity and affect the entry of peripheral immune cells into the CNS. Therefore, the expression of MMPs is tightly regulated at the level of gene transcription, conversion of pro-enzyme to active MMPs and by the action of TIMP (Rosenberg, 2002). While the increase in MMP-9 levels as observed by us supports the previous studies, demonstrating its increase by WNV, JEV and VEEV (Mishra et al., 2009; Schafer et al., 2011; Wang et al., 2008a), the unique aspect of our data is the increase of MMP-1 and -3 in the brains of WNV-infected mice. The increase of MMP-3 was at a greater extent compared with MMP-9 in the brain. This result is in agreement with our previous in vitro studies, demonstrating upregulation of MMP-1, -3 and -9 in WNV-infected human astrocytes (Verma et al., 2010). Based on several novel roles of MMP-1 and -3 in mediating neuroinflammation and apoptosis (Kim et al., 2005; Mun-Bryce et al., 2002; Suzuki et al., 2007), this observation warrants further delineation of the role of other MMPs in WNV neurological disease. In our study, the increased MMP levels coincided with increased virus titres in the CNS (Fig. 1), activation of astrocytes (Fig. 3), BBB disruption and decreased TJP expression (Fig. 2). Based on our previous observation (Verma et al., 2010) as well as recent report of MMP-9 production by JEV-infected astrocytes (Tung et al., 2010), it seems likely that activated astrocytes might be one of the potential sources of these MMPs in the brain, which participate in the remodelling of the BBB by degrading junctional complex proteins. However, the possibility of infiltrating immune cells as another source of MMPs production at day 8 after infection cannot be ruled out.

WNV replication in the CNS plays a critical role in mediating BBB disruption

In the mouse model of WNV disease, it is believed that WNV invades the CNS when viraemia is high and is first detected in the CNS between days 4 and 6 after infection (Klein & Diamond, 2008). During this period, WNV can enter the CNS via multiple routes, including by crossing the BBB. Our results indicate that in spite of high viraemia and increased MMP-9 levels in the serum there were no detectable changes in the BBB permeability between days 2 and 4 after infection. On the other hand, the most dramatic change in BBB integrity was observed at day 8 after infection when the virus titres and MMP levels were significantly elevated in the brain. These results suggest that the initial entry of infectious West Nile virions into the CNS between days 4 and 6 after infection might occur without altering the BBB integrity by one or more of the proposed routes of virus entry such as directly infecting BBB-endothelial cells (Samuel et al., 2007; Verma et al., 2009). Virus replication in the CNS then induces inflammation, including production of multiple MMPs and cytokines, which causes BBB disruption. To further confirm the hypothesis of BBB disruption being driven by inflammatory mediators produced in the brain, we infected mice intracranially with WNV. In favour of this notion, we observed a similar increase in BBB disruption as measured by the presence of IgG and MMPs in the brain at day 6 after infection (Fig. 7). The pathophysiological importance of BBB disruption at later time points supports the hypothesis that enhanced infiltration of activated immune cells into the brain, some of which may be infected, may facilitate a second wave of virus neuroinvasion via the ‘Trojan-horse’ route. Recent studies using Drak2−/− mice have emphasized this phenomenon, by demonstrating that infected infiltrating T-cells can be potential carriers for WNV entry into the CNS (Wang et al., 2008b). As observed
by us (Fig. 5) and others (Lim et al., 2011), increased leukocyte infiltration in the infected brain beginning at day 8 after infection further supports the fact that BBB disruption might facilitate unrestricted entry of peripheral immune cells into the CNS. A similar argument, that virus replication in the brain induces BBB opening allowing a second wave of invading virus, has been recently proposed for VEEV (Schafer et al., 2011). Our data do not explain the relationship of BBB disruption with overall disease outcome; however, they do indicate an important role of BBB disruption in the increased transmigration of activated infected and/or naïve immune cells into the brain. Inhibition of BBB opening may not block the initial invasion of the virus, but it is likely to reduce the neuropathology associated with unrestricted entry of virus and activated infected and/or naïve immune cells in the CNS. Although virus specific T-cells are critical for WNV clearance from the brain, enhanced infiltration of immune cells as a result of BBB disruption may exacerbate neuronal damage by potentiating neuroinflammation, resulting in poor disease prognosis.

In conclusion, this first in vivo study demonstrates dramatic alterations in the expression kinetics of junction proteins of the brain vascular endothelial cells following WNV infection in mice. The kinetics of TJP and AJP degradation follow closely the pattern of BBB disruption during the course of WNV infection. Furthermore,
concomitant increase of multiple MMPs in the CNS of mice inoculated either in the footpad or directly into the cranium indicates that MMPs derived from the brain play a critical role in TJP degradation. Overall these findings suggest that BBB-tight junction disruption is a critical pathological event in WNV neuropathogenesis that may permit the paracellular transit of virus and immune cells into the CNS. Future studies of blocking BBB disruption are warranted to further delineate the regulatory mechanisms underlying WNV-induced BBB disruption, to develop therapeutic modalities to ameliorate the pathology associated with WNV neuropathogenesis.

METHODS

Virus and mouse experiments. Wild-type C57BL/6 mice bought from the Jackson Laboratory were bred in the animal facility at the John A. Burns School of Medicine. All experiments were approved and conducted in accordance with the University of Hawaii at Manoa animal study guidelines. All infections were conducted on 8–10-week-old mice by inoculation of 100 p.f.u. (LD₅₀) of virus (NY99 isolated from crow brain passaged once in Vero cells) either in the footpad or cranium as described previously (Verma et al., 2011). Blood was collected by cardiac puncture and serum was separated by centrifugation and was stored at -80°C. Mice were then perfused with 20 ml cold PBS under anaesthesia and harvested organs, after flash freezing, were stored at -80°C.

Assay of BBB integrity. BBB integrity was evaluated by using the Evans blue dye exclusion test and by measuring IgG extravasation in the brain (Wang et al., 2008a). Control and infected mice were injected in the peritoneal cavity with 1 ml Evans blue dye [1% (w/v) in PBS]. One hour later, mice were perfused with 20 ml PBS, euthanized under anaesthesia and the brains and peripheral tissues were harvested and photographed. Western blotting was conducted by using total protein extract of the brain to detect heavy and light chains of IgG.

Quantification of viral load in blood and tissues. Virus titres were analysed by plaque assays and qRT-PCR in the serum and brain, respectively, as described previously (Verma et al., 2011). qRT-PCR was conducted using primers and FAM- and TAMRA-labelled probes specific for WNV env region and the standard curve was generated by using RNA extracted from previously titrated WNV dilutions (10⁰⁰ to 0.1 p.f.u.) as described by Lanciotti et al. (2000). The data are expressed as WNV p.f.u. equivalents µg⁻¹ of RNA.

qRT-PCR determination of host genes. The mRNA levels of multiple TJP, AJP and MMPs were determined by using qRT-PCR, and the fold change in infected brains as compared to controls was calculated after normalizing to the GAPDH gene (Verma et al., 2010). The primer sequences and annealing temperatures used for qRT-PCR are listed in Table S1 (available in JGV Online).

Western blot analysis. Total cellular protein was extracted by sonicating the brain in chilled Cell Lytic buffer (Sigma) followed by centrifugation at 11,000 g for 20 min and the soluble supernatant fraction was collected. An equal amount of total protein (25–40 µg) was separated by SDS-PAGE, transferred onto nitrocellulose membrane and incubated overnight with polyclonal antibodies against claudin-1, occludin, ZO-1 (Zymed), VE-cadherin, JAM-A (Santa Cruz), β-catenin (Abcam) and β-actin (Sigma) as described previously (Verma et al., 2010). Following incubation with secondary antibodies conjugated with IRDye 800 and IRDye 680 (Li-Cor Biosciences), the membranes were scanned by using the Odyssey

Fig. 7. Intracranial inoculation of WNV results in BBB disruption. (a) WNV titres in the brain of mice intracranially inoculated with 100 p.f.u. WNV. Brain titres were determined by qRT-PCR and expressed as p.f.u. µg⁻¹ RNA. (b) Leakage of IgG in the brain was determined by Western blotting. (c) MMP-1, -3 and -9 mRNA transcripts increased in the brains of intracranially inoculated mice as determined by qRT-PCR. Data represents at least three mice per time point.
infrared imager (Li-Cor Biosciences). Intensity of bands for densitometric analysis was determined by using the Odyssey program software.

**Measurement of MMPs using ELISA.** The protein levels of MMP-3 and -9 in mouse brain homogenates and sera were measured using commercial ELISA kits (R&D) following the instruction manual as described previously (Verma et al., 2010).

**Immunohistochemistry.** Mice were transcardially perfused with 20 ml PBS followed by 20 ml of 4 % paraformaldehyde (PFA), brains were harvested, cryoprotected in 30 % sucrose (Sigma) for 3 days at 4 °C and frozen in Optimal Cutting Temperature solution (Tissue-Tek). Horizontal sections of 10 μm thickness were fixed in 2 % PFA for 15 min, permeabilized by incubating 0.3 % Triton X-100 (Sigma) in PBS for 1 h at room temperature and then blocked for 1 h using 5 % goat serum (Jackson ImmunoResearch) in PBS. The staining with various primary antibodies (GFAP, vWF, claudin-1, occludin, ZO-1, JAM-A, VE-cadherin and β-catenin) was conducted overnight at 4 °C, followed by incubation with biotinylated secondary antibodies for 2 h at room temperature and developed by using fluorescein labelled streptavidin conjugate. Images were acquired by using the fluorescent microscope (Zeiss Axiovert 200). Tissue sections were also stained with haematoxylin and eosin and examined for pathological changes by using a Nikon E600 phase-contrast microscope.

**Preparation of cells and staining for flow cytometry.** Single-cell suspensions of the brains were prepared by using MACS Neural Tissue Dissociation kit and GentleMACS cell dissociator system (Miltenyi Biotec). The suspension was brought to 10 ml in 30 % Percoll with FACS buffer and overlaid on 1 ml 70 % Percoll. After centrifuging at 700 g for 30 min, leukocytes at the interphase were isolated, washed, blocked in 200 μl Fc block (CD16/32) and stained with anti-CD11b-PE-Cy7 for 30 min and analysed using FACS Aria (BD) and FlowJo software.

**Statistical analysis.** All mRNA and protein quantification data are reported as SEM. Either unpaired Student’s t-test or Mann–Whitney U test for single-mean comparison was conducted using GraphPad Prism 5.0 (GraphPad software). P<0.05 was considered as statistically significant for all analyses.

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