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Original research

Engagement of AKT and ERK signaling pathways facilitates infection of human neuronal cells with West Nile virus

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ABSTRACT

West Nile virus (WNV) is an important neurotropic virus that accounts for the emergence of human arboviral encephalitis and meningitis. The interaction of WNV with signaling pathways plays a key role in controlling WNV infection. We have investigated the roles of the AKT and ERK pathways in supporting WNV propagation and modulating the inflammatory response following WNV infection. WNV established a productive infection in neuronal cell lines originated from human and mouse. Expression of IL-11 and TNF-α was markedly up-regulated in the infected human neuronal cells, indicating elicitation of inflammation response upon WNV infection. WNV incubation rapidly activated signaling cascades of AKT (AKT-S6-4E-BP1) and ERK (MEK-ERK-p90RSK) pathways. Treatment with AKT inhibitor MK-2206 or MEK inhibitor U0126 abrogated WNV-induced AKT or ERK activation. Strong activation of AKT and ERK signaling pathways could be detectable at 24 h after WNV infection, while such activation was abolished at 48 h post infection. U0126 treatment or knockdown of ERK expression significantly increased WNV RNA levels and viral titers and efficiently decreased IL-11 production induced by WNV, suggesting the involvement of ERK pathway in WNV propagation and IL-11 induction. MK-2206 treatment enhanced WNV RNA replication accompanied with a moderate decrease in IL-11 production. These results demonstrate that engagement of AKT and ERK signaling pathways facilitates viral infection and may be implicated in WNV pathogenesis.

1. Introduction

West Nile virus (WNV), an important member of flaviviruses, contributes to arboviral neuroinvasive diseases. It is considered to be the most widespread arbovirus for its expanding geographic distribution, including several arboviral neuroinvasive disease outbreaks.[1] WNV is designated into at least 5 phylogenetic lineages, with lineage 1 and 2 viruses associated with the outbreaks in humans. Its endemic zone has spread from Africa, Europe, and Australia to Asia.[2] In nature, WNV is maintained in a bird-mosquito-bird transmission cycle. Birds are the natural reservoirs, and several vertebrate species such as horses and humans are incidental hosts.[3] Culex mosquitoes are the primary vectors. The host-vector-WNV interaction has resulted in thousands of cases in birds, horses, and humans.[4] Due to the progressive changing host, vector, and environmental conditions, WNV poses a significant threat to public health worldwide.[4]

Flaviviruses are globally distributed human pathogens that are responsible for up to 400 million infections annually, leading to various diseases such as hepatitis, vascular shock syndrome, encephalitis, and acute flaccid paralysis.[5,6] Among these diseases, viral meningitis and encephalitis are increasingly diagnosed, with limited antiviral therapies and vaccines. Arboviruses including WNV, Japanese encephalitis virus, tick-borne encephalitis virus, and Zika virus are the most common causes of viral meningitis and encephalitis.[7] WNV infection leads to clinical symptoms ranging from a mild fever to meningitis, encephalitis, and death, with development of long-term neurological sequelae.[8] It can also causes chronic kidney disease.[9] Neither licensed vaccines nor approved antiviral drugs are available for humans to combat this infection.[10] Neuropathological features of WNV highlight the extent of viral damage occuring in the central nervous system (CNS). The molecular mechanisms of WNV neuropathogenicity remain to be pursued due to the lack of effective prevention and treatment strategies.

The immune response is critical for controlling WNV replication, limiting its dissemination and CNS invasion. The interactions between WNV and the immune response are closely linked to its clearance from the CNS, which accounts for its neuropathology.[11] A variety of cytokines

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and chemokines are regarded as neuroinflammatory factors that are involved in pro and anti-inflammatory effects. Some cytokines offer protection against acute WNV infection and facilitate viral clearance, while others play multifaceted roles in WNV neuropathogenesis and inflammation-mediated tissue damage obtained from the murine model of WNV infection.[12] Although progress in dissecting WNV-host factors interactions has been made, efforts are still needed to identify the pivotal cellular processes controlling WNV infection and inflammation response.

Upon viral infection, the signaling pathways resulting in type I interferon (IFN) are elicited to combat viruses first defense line. Other pathways are triggered by viruses to help them establish productive infection through prevention of viral clearance, induction of viral spread, and promotion of viral propagation. There are many efforts looking at interferon (IFN) signaling to elucidate WNV pathogenesis. Type I IFN signaling has been shown to induce the defense response by limiting WNV replication and spread.[13] WNV has evolved strategies to evade immune response by targeting various aspects of the IFN signaling, leading to evasion and disruption of the innate immune response.[14] It has been demonstrated that WNV prevents phosphorylation of Janus kinases JAK1 and Tyk2 and that IFN signaling is antagonized by WNV nonstructural protein 5.[15,16] WNV nonstructural protein 1 inhibits Toll-like receptor 3 signaling and antagonizes IFN-beta production.[17,18]

The mechanisms underlying WNV-mediated neuronal damage are not well understood. The ability of WNV to modulate cellular response by targeting signaling pathways is a key in controlling WNV infection and disease outcome. In the present study, an association of signaling events initiated by WNV and its propagation and pathogenesis was investigated.

2. Materials and methods

2.1. Cell culture

Human neuroblastoma cells SH-SY5Y (ATCC CRL-2266), glioblastoma cells U251 (Cell bank of Chinese Academy of Sciences, Shanghai, China), mouse neuroblastoma cells Neuro-2a (ATCC CCL-131), African green monkey kidney cells Vero (ATCC CCL-81), and baby hamster kidney cells BHK-21 (ATCC CCL-10) were cultured. SH-SY5Y, U251, and Neuro-2a cells were used for WNV infection. Vero cells were used to propagate WNV. BHK-21 cells were used for plaque assay. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 1% L-glutamine, 1% non-essential amino acid solution, 1% penicillin-streptomycin, and 10% fetal bovine serum at 37 °C in a humidified incubator with 5% CO_2.

2.2. WNV production

Working stocks of WNV[19] were produced in Vero cells, and culture supernatants collected at 72 h post incubation were centrifuged at 2500 rpm for 10 min to remove cell debris. Aliquots of culture supernatants were stored at −80 °C until use. Stock titer (multiplicity of infection [MOI]) was determined by plaque assay. Culture supernatants of Vero cells were harvested at 72 h and referred to as mock inoculum. Experiments concerning WNV production and infection were performed at Biosafety Level-3 in accordance with the guidelines by the Committee on Safety of Biomedicine at the Naval Medical University.

2.3. WNV infection

Cells were seeded in 35 mm dishes and cultured overnight to form a confluent monolayer. For WNV incubation, cells were incubated with the virus for 5, 15, 30, and 60 min at 37 °C, respectively. For WNV infection, cells were incubated with virus in culture medium for 1 h of adsorption at 37 °C and that unbound virus was washed away with phosphate-buffered saline (PBS). Cells were then maintained in fresh culture medium for durations of 12, 24, and 48 h. Time points were measured from the end of the 1 h adsorption. Incubation or infection of cells was carried out with 2 MOI of WNV throughout this study. At the indicated time points, culture supernatants, cellular RNA, and cell lysates were collected and prepared for measurement of WNV, cytokines, and signal molecules, respectively.

2.4. Viability assay

SH-SY5Y cells seeded in 96-well plates were cultured overnight to form a confluent monolayer. Cells were cultured for 24 h in fresh culture medium containing various concentrations of MEK1/2 inhibitor U0126 (Cell Signaling Technology) or AKT inhibitor MK-2206 (Selleck) dissolved in dimethylsulfoxide (DMSO; Sigma). Cell viability was evaluated using CellTiter 96®AQueous One Solution Cell Proliferation kit (Promega) following the manufacture's instructions. The absorbance at 490 nm was read on a Synergy 2 Multi-Mode Microplate Reader (BioTek).

2.5. Inhibitor treatment

To assess influence of the inhibitors on the signaling pathways triggered by WNV, SH-SY5Y cells seeded in 12-well plates were cultured overnight to form a confluent monolayer. Cells were pretreated for 1 h with increasing concentrations of MEK1/2 inhibitor U0126 (0, 5, and 10 μM) or AKT inhibitor MK-2206 (0, 1, and 5 μM), washed twice with PBS, and incubated with WNV for 5 min at 37 °C.

To analyze the influence of the inhibitors on WNV propagation and cellular response, SH-SY5Y cells in 12-well plates at a confluent monolayer were pretreated with the U0126 or MK-2206 for 1 h at 37 °C, washed twice with PBS, incubated for 1 h with WNV. After two washes with PBS, cells were maintained in fresh culture medium containing the U0126 or the MK-2206 for 24 h.

2.6. Transient transfection and WNV infection

SH-SY5Y cells seeded in 12-well plates at approximately 50% confluence were transfected with ERK1/2 siRNA (Silencer ERK1/2 siRNA) or control siRNA (Silencer negative control siRNA, Cell Signaling Technology) at a final concentration of 100 nM using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were maintained for 72 h in culture medium without antibiotics. After removing the medium, the cells were incubated with WNV for 1 h, washed twice with PBS, and allowed to proceed for additional 24 h culture.

2.7. Plaque assay

BHK-21 cells seeded in 60 mm dishes were grown overnight to form a confluent monolayer. Cells were inoculated for 1 h with 10-fold dilutions of cell culture supernatants containing WNV at 37 °C. After removal of the virus inoculum, the cells were washed once with PBS and 2% agar overlay medium was added onto the cells. Following a 4-day incubation, the cells were stained with 0.02% neutral red to visualize plaques. Calculation of plaque-forming units (PFU) was carried out.

2.8. Western blot analysis

Cells were washed with PBS and lysed in blue loading buffer containing dithiothreitol (Cell Signaling Technology) following the manufacturer's protocol. Proteins in cell lysates were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene difluoride membranes using a Mini-Protein II system (Bio-Rad). After incubating for 2 h with 5% non-fat milk blocking buffer, membranes were incubated at 4 °C overnight with rabbit primary antibodies for AKT, S6 ribosomal protein, 4E-BP1,

MEK1/2, ERK1/2, phospho-AKT (Ser473), phospho-S6 ribosomal protein (Ser235/236), phospho-4E-BP1 (Thr70), phospho-MEK1/2 (Ser217/221), phospho-ERK1/2 (Thr202/Tyr204), phospho-p90RSK (Ser380), or β-actin (Cell Signaling Technology). Membranes were incubated for 2 h with horseradish peroxidase-linked goat anti-rabbit antibody. Protein bands were detected with enhanced chemiluminescent solution (Bio-Rad) on a GeneGnome HR image capture (Cambridge). Quantitative analysis of protein bands was carried out using GeneTools software.

2.9. Real time reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted using TRIzol™ (Invitrogen) according to the manufacturer's instructions. Reverse transcription on 2 μg of extracted RNA was preformed using random primers and moloney murine leukemia virus reverse transcriptase kit (Promega). Real time quantitative PCR assay was performed on the cDNA generated using SYBR Green PCR kit (Promega). The primer sequences for WNV, interleukin-11 (IL-11), tumor necrosis factor alpha (TNF-α), activating transcription factor 6 (ATF6), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were listed in Table S1. The signals were acquired on a Rotor-Gene 3000 Thermal Cycler (Corbett). For target gene expression analysis, relative expression values were normalized to the GAPDH internal control and calculated using the threshold cycle ($2^{-\Delta\Delta CT}$) method (Rotor-Gene 6.1.81 software).

2.10. IL-11 measurement

Enzyme-linked immunosorbent assay (ELISA) was carried out to test IL-11 release in culture supernatants from SH-SY5Y cells. IL-11 amounts were determined using Quantikine human IL-11 ELISA kit (R&D Systems) following the manufacturer's protocol. Data were acquired on the Synergy 2 Multi-Mode Microplate Reader and analyzed by SigmaPlot 10.0 software.

2.11. Statistical analysis

Data are expressed as means and standard deviations. Two-tailed unpaired or paired Student's *t*-test (GraphPad Prism 8.0 software) was used to determine statistical differences. Statistically significant differences were considered for $^{*}P<0.05$, $^{**}P< 0.005$, and $^{***}P<0.001$.

3. Results

3.1. Establishment of WNV infection in neuronal cell lines originated from human and mouse

Cell cultures and animal models have been applied to elucidate WNV pathogenesis. Due to the limitation of primary human neuronal cells, neuronal cell lines are utilized as experimental models. Human SH-SY5Y neuroblastoma cells were frequently used in neurobiology and described to be a useful neuronal cell model for development of pharmacological agents.[20,21] Human U251 cells were a common model of glioblastoma and used to elucidate neuroprotective effects of verbascoside against Alzheimer's disease.[22,23] Mouse Neuro-2a neuroblastoma cells was a tool to study neurotropic behavior of Chandipura virus.[24]

In the present study, the neurotropic behavior of WNV was characterized in SH-SY5Y, U251, and Neuro-2a cells based on WNV RNA replication and virus production. Kinetics of WNV RNA replication was explored in cells infected with WNV for the various time periods by real time PCR analysis. WNV RNA was only detectable in the SH-SY5Y and U251 cells at 12 h post infection (Fig. 1A–C). WNV RNA levels were

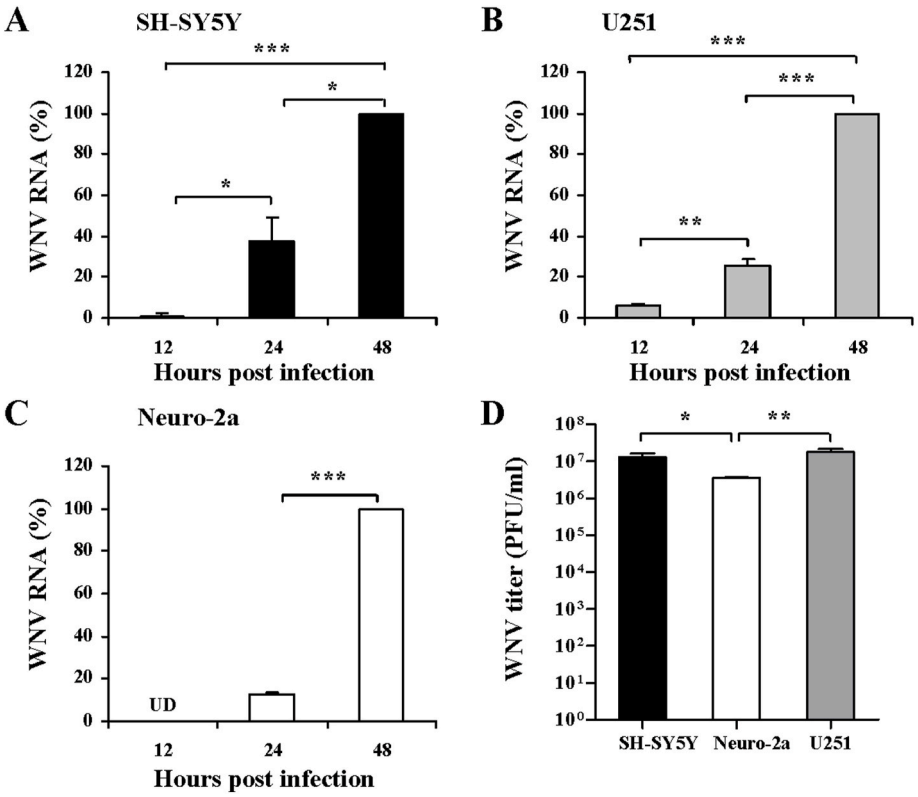

Fig. 1. Multiple neuronal cell lines supported West Nile virus (WNV) infection. WNV RNA levels in virus-infected human SH-SY5Y (A), U251 (B), and mouse Neuro-2a (C) cells. Cells were infected with WNV and cellular RNA was extracted at 12, 24, or 48 h post infection. WNV RNA levels were quantified by real time RT-PCR on the extracted RNA. WNV RNA levels are shown as relative percentages of the cells infected with WNV for 48 h. (D) WNV propagation in the cell lines. Culture supernatants from the SH-SY5Y, U251, and Neuro-2a cells were harvested at 48 h post infection and WNV titers were detected by plaque assay. Data are representative of three experiments performed in triplicate.$^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$. UD, undetectable.

significantly increased at 24 h post infection as compared with those at 12 h (for SH-SY5Y, $P<0.05$; for U251, $P<0.005$), with detectable WNV RNA in the Neuro-2a cells, implying that WNV replication efficiency varied among cell lines. At 48 h post infection, levels of WNV RNA were higher in the three cell lines than those at 24 h (for SH-SY5Y, $P<0.05$; for U251, Neuro-2a, $P<0.001$), indicating a time-dependent increase in WNV RNA replication. Plaque assay was thereby performed to assess WNV production at 48 h post infection. In comparison with WNV titers in the Neuro-2a cells, the viral titers were significantly increased in the SH-SY5Y ($P<0.05$) and U251 ($P<0.005$) cells (Fig. 1D). Thus, WNV established a productive infection and displayed differences in replication efficiency among neuronal cells. These data enabled us to investigate the inflammatory response upon WNV infection.

3.2. Inflammatory response elicited by WNV in human neuronal cells

The understanding of inflammatory response upon WNV infection in the CNS provided new insights into the pathogenesis of WNV-induced neurological manifestations.[25] To assess the inflammatory response elicited by WNV, mRNA levels of multiple cellular genes involved in endoplasmic reticulum stress (ATF6) and inflammation response (pro-inflammatory cytokine TNF-α; anti-inflammatory cytokine IL-11) were measured in SH-SY5Y and U251 cells infected with WNV for varying time periods. Relative mRNA expression of IL-11, TNF-α, and ATF6 was detected by real time PCR analysis. Fig. 2A show that mRNA expression of IL-11 and TNF-α was up-regulated in infected SH-SY5Y cells. In comparison with mRNA levels at 12 h post infection, mRNA expression of IL-11 and TNF-α was significantly increased at 24 h ($P<0.05$) or 48 h (for IL-11, $P<0.005$; for TNF-α, $P<0.001$). Conversely, ATF6 mRNA expression was significantly decreased in infected SH-SY5Y cells ($P<0.05$). The mRNA expression of IL-11 and TNF-α was up-regulated by WNV in a time-dependent manner, with down-regulation of the ATF6 mRNA expression.

Similarly, WNV infection enhanced IL-11 and TNF-α mRNA expression in U251 cells (Fig. 2B). In comparison with mRNA levels at 12 h post infection, mRNA expression of IL-11 and TNF-α was significantly up-regulated at 24 h ($P<0.05$) or 48 h (for IL-11, $P<0.05$; for TNF-α, $P<0.001$). As for ATF6, there were no significant changes in mRNA expression upon WNV infection. In response to WNV infection, mRNA expression of IL-11 and TNF-α was up-regulated, whereas such up-regulation was not detectable on ATF6 mRNA expression in the SH-SY5Y and U251 cells, suggesting that different expression profiles of host factors are associated with stages of WNV infection and may be responsible for neuroinflammation. Moreover, the up-regulation of IL-11 and TNF-α mRNA expression was consistent with the kinetics of WNV replication in the SH-SY5Y and U251 cells (Fig. 1A–B), indicating that the inflammation response is in parallel with WNV replication. Together with the productive infection of WNV (Fig. 1D), elucidation of WNV neuropathogenesis was pursued in SH-SY5Y cells.

3.3. AKT and ERK signaling pathways triggered by WNV incubation

Some signaling pathways involved in triggering immune defenses have been highlighted during WNV infection.[14] Due to the importance of the AKT and ERK pathways in fundamental biological processes, we have addressed the AKT and ERK pathways in SH-SY5Y cells infected with WNV for various time periods. The signaling cascades were monitored by Western blot analysis of the total and phosphorylated

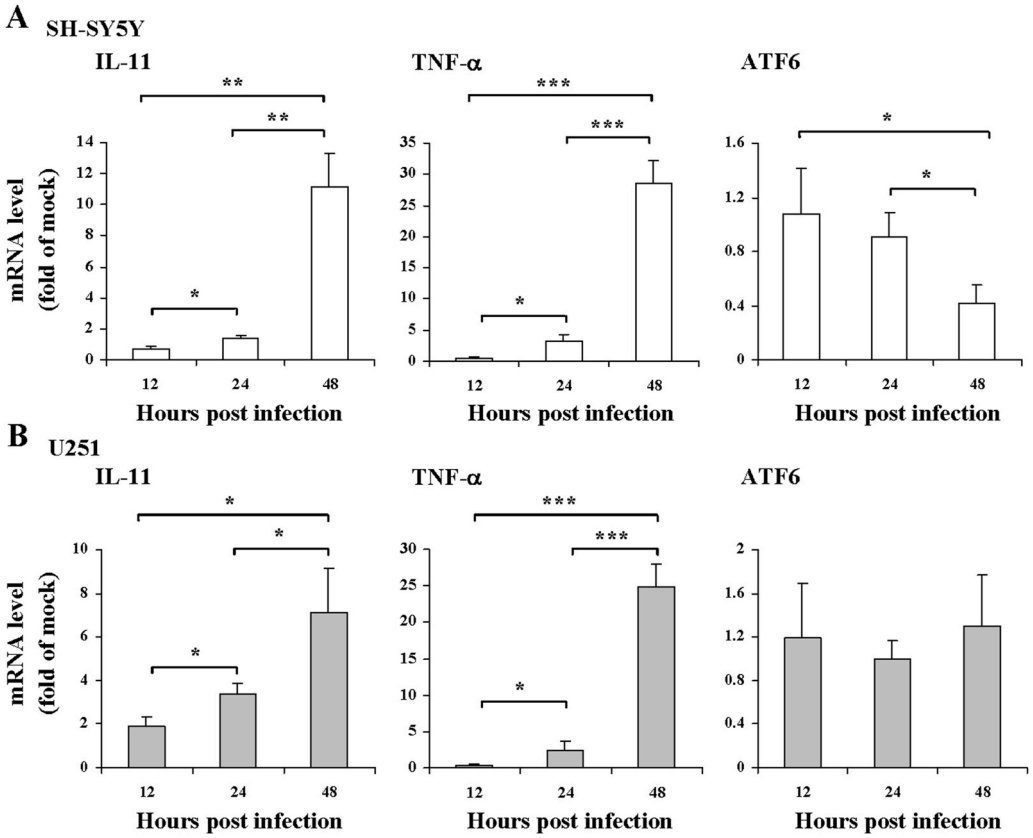

Fig. 2. West nile Virus (WNV) infection regulated cellular gene expression in human neuronal cell lines. IL-11, TNF-α, and ATF6 gene expression in SH-SY5Y (A) and U251 (B) cells upon WNV infection. Cells were infected with WNV and cellular RNA was extracted at 12, 24, or 48 h post infection. The mRNA levels of IL-11, TNF-α, and ATF6 were quantified by real time RT-PCR. Data are shown as relative fold of the mRNA levels in the cells infected with WNV over the corresponding levels in the cells incubated with the mock inoculum (mock) at the indicated time points. Data are representative of three experiments performed in triplicate. *$P<0.05$, **$P<0.005$, ***$P<0.001$.

kinases of the AKT and ERK pathways. The kinase phosphorylation represents its activation status. AKT phosphorylation was rapidly increased after WNV incubation and peaked at 5 min (Fig. 3A). WNV-induced AKT phosphorylation decayed rapidly and disappeared at 60 min. Upon WNV incubation, phosphorylation of downstream S6 and 4E-BP1 was induced at 5 min, peaking at 15 min, decaying at 30 min, and diminishing by 60 min. The total kinases were almost unaffected by the WNV incubation. We wondered whether the profiles of AKT signaling triggered by WNV could be observed in other cell lines. The AKT signaling cascades were further evaluated in Vero cells that were used to propagate WNV. In Vero cells, AKT was slightly phosphorylated and activated during 5–60 min of WNV incubation, without S6 activation (Fig. S1A). Cells incubated with the culture supernatants of Vero cells were used as a mock control. There are differences in the patterns of AKT signaling pathway triggered by WNV in different cell types.

As for the ERK pathway, phosphorylation of sequential kinases MEK1/2, ERK1/2, and p90RSK was analyzed in response to WNV incubation. Rapid and strong activation of the ERK pathway was detectable in the SH-SY5Y cells. MEK1/2 phosphorylation peaked at 5 min of WNV incubation and activation was sustained by 60 min (Fig. 3B). Similarly, the 5 min of WNV incubation led to peak levels of ERK1/2 and p90RSK phosphorylation and activation sustained by 60 min. Similar results were obtained with phosphorylation of MEK1/2, ERK1/2, and p90RSK in Vero cells (Fig. S1B). These results implied a universal regulation of ERK signaling by WNV. The WNV-activated AKT pathway appeared transiently with a rapid peak and disappearance. The WNV-activated ERK pathway peaked rapidly with strong activation which was maintained longer. The distinctive regulation of AKT and ERK pathways may be critical for understanding the early signaling events involved in WNV infection.

3.4. Inhibition of WNV-induced AKT and ERK1/2 activation by MK-2206 or U0126

To explore whether the activation above is specific to the WNV incubation, SH-SY5Y cells were treated with inhibitors at increasing concentrations prior to WNV incubation. Fig. 3C show that the AKT phosphorylation induced by WNV was inhibited by the AKT inhibitor MK-2206 in a concentration-dependent manner. The treatment with 5 μM MK-2206 abolished the AKT activation upon WNV incubation. ERK1/2 phosphorylation induced by WNV was also inhibited by the MEK1/2 inhibitor U0126 in a concentration-dependent manner (Fig. 3D). Treatment with 10 μM U0126 markedly inhibited the ERK1/2 activation upon WNV incubation. There were no obvious inhibitory effects on AKT and ERK1/2 activation in the cells treated with DMSO (dissolvent control). Indeed, the AKT and ERK pathways were activated due to the WNV incubation.

3.5. Dynamic regulation of AKT and ERK signaling pathways by WNV infection

The status of signaling pathways may be varied in response to viral infection for various time periods, which represents the host-virus interaction and accounts for the cellular response and viral

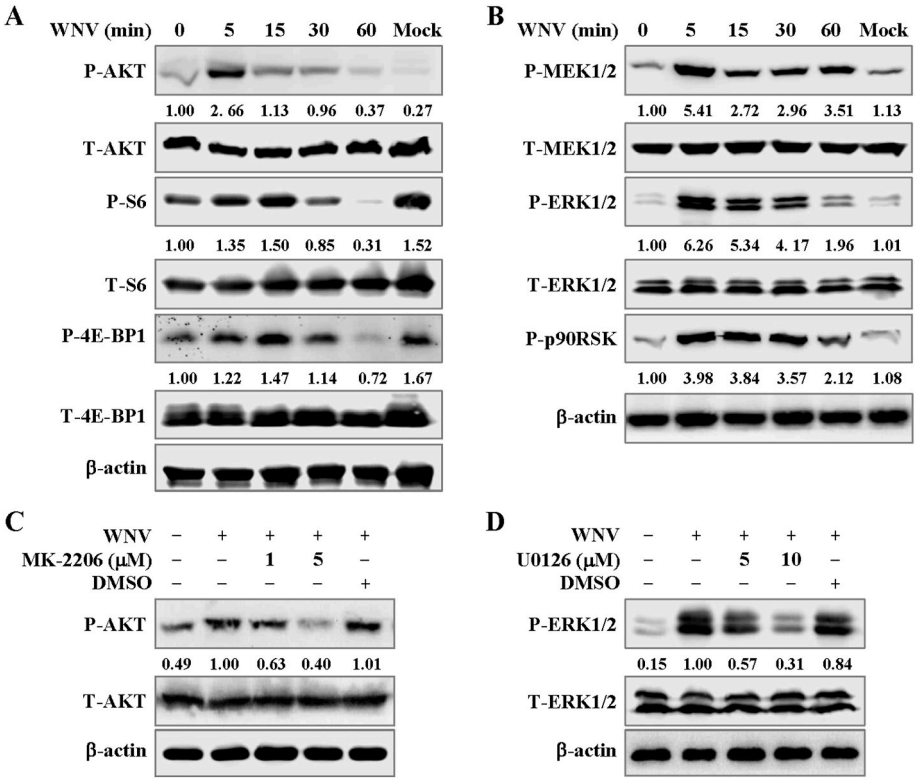

Fig. 3. West Nile virus (WNV) incubation triggered AKT and ERK signaling pathways in SH-SY5Y cells. (A) Kinetics of AKT, S6, and 4E-BP1 phosphorylation mediated by WNV incubation. (B) Kinetics of MEK1/2, ERK1/2, and p90RSK phosphorylation mediated by WNV incubation. Cells were incubated with WNV for 0 (unincubated), 5, 15, 30, or 60 min and cell lysates were prepared. Western blot analysis was performed on the cell lysates using total (T-) or phosphorylated (P-) forms of antibodies for AKT, S6, 4E-BP1, MEK1/2, ERK1/2, or p90RSK. Fold changes of phosphorylated protein levels in the cells incubated with WNV or the mock inoculum (mock) over those in the unincubated cells are shown below each blot after being normalized to β-actin levels. (C) Inhibition of WNV-mediated AKT phosphorylation by MK-2206. (D) Inhibition of WNV-mediated ERK1/2 phosphorylation by U0126. Cells were pretreated with the inhibitors or DMSO dissolvent and incubated with WNV. Total (T-) and phosphorylated (P-) forms of AKT and ERK1/2 were detected. β-actin is shown as a loading control. Fold changes of phosphorylated AKT and ERK1/2 levels in the cells pretreated with the inhibitors or DMSO over those in the WNV-incubated cells are shown below each blot after being normalized to β-actin levels. The results are representative of three experiments. −, without; +, with.

pathogenesis. The AKT and ERK signaling pathways were further explored in SH-SY5Y cells infected with WNV for long time periods. Phosphorylated and total kinases were assessed at the indicated time points. Levels of AKT phosphorylation were increased at 12 h or 24 h post infection as compared with mock-incubation, whereas AKT phosphorylation was almost diminished at 48 h post infection (Fig. 4A). Similarly, phosphorylation levels of the downstream S6 and 4E-BP1 were also increased at 12 h or 24 h post infection, and such phosphorylation was diminished at 48 h. In addition, phosphorylation of AKT and S6 was obviously inhibited in Vero cells at 48 h post infection (Fig. S2). The results showed the early activation and later suppression of the AKT pathway signaling following WNV infection over the experiment periods.

The kinetics of ERK signaling by WNV infection was shown in Fig. 4B. In comparison with phosphorylation of MEK1/2 and ERK1/2 in the mock-incubated cells, there were no obvious differences in the MEK1/2 and ERK1/2 phosphorylation at 12 h post infection. WNV infection led to strong phosphorylation of MEK1/2 and ERK1/2 at 24 h, and abolished such phosphorylation at 48 h. Phosphorylation of p90RSK was enhanced at 12 h, peaked at 24 h, and declined at 48 h post infection. In Vero cells, phosphorylation of MEK1/2, ERK1/2, and p90RSK was also markedly inhibited or abolished by WNV at 48 h post infection (Fig. S2). Therefore, the patterns of AKT and ERK signaling pathways were differentially regulated by WNV infection. These observations suggest that WNV could interfere with signaling of the AKT and ERK pathways. Further experiments were carried out to investigate whether such regulation correlated with WNV propagation and cellular response.

3.6. Involvement of the AKT and ERK pathways in WNV propagation

The contributions of the AKT and ERK signaling pathways for WNV propagation were evaluated by the use of inhibitors for AKT or MEK1/2. The impact of the inhibitors on cell viability were first assessed. As shown in Fig. 5A, U0126 was nontoxic to SH-SY5Y cells at concentrations up to 10 μM and exerted significant cytotoxic effect at 20 μM ($P<0.05$). At a concentration of 0.5 μM or 1 μM, MK-2206 induced a slight decrease in cell viability in that cell viability was reduced to 73% upon 5 μM MK-2206 treatment. Then, the effect of inhibitors on AKT and ERK1/2 phosphorylation was examined. Western blot analysis showed that WNV-mediated ERK1/2 phosphorylation was blocked by 10 μM U0126 (Fig. 5B). Similarly, WNV-mediated AKT phosphorylation was abolished with 1 μM MK-2206 treatment (Fig. 5C). Total ERK and AKT seemed to be unaffected upon the treatment of inhibitors. The ERK and AKT signaling was blocked by inhibitors in WNV-infected SH-SY5Y cells.

Next, WNV propagation was assessed in the infected cells treated with the inhibitors or left untreated. WNV-infected cells treated with DMSO were used as a dissolvent control. Fig. 5D showed that WNV RNA levels were significantly increased in the cells treated with 10 μM U0126 or 1 μM MK-2206 ($P<0.05$) as compared with those in the untreated cells. In comparison with WNV RNA levels in the DMSO-treated cells, viral RNA levels were also significantly increased in cells treated with the inhibitors (for U0126, $P<0.005$; for MK-2206, $P<0.05$). At the same time, plaque assay was performed to determine WNV production in the culture supernatants from infected cells with and without inhibitor treatment. Fig. 5E showed that WNV titers were significantly increased in the cells treated with 10 μM U0126 as compared with those in untreated ones as well as the DMSO-treated cells ($P<0.05$). The 1 μM of MK-2206 treatment only exerted a slight increase in viral titers. These results showed that inhibition of the ERK signaling led to the enhancement of WNV replication and production.

Moreover, down-regulation of ERK1/2 expression in WNV-infected SH-SY5Y cells was applied to evaluate the influence of ERK1/2. As shown in Fig. 6B, WNV RNA levels were significantly increased in cells with the ERK1/2 siRNA transfection as compared with those in untransfected ones as well as the control siRNA transfected ones ($P<0.05$). In accordance with viral RNA levels, WNV titers were also significantly increased in cells transfected with the ERK1/2 siRNA as compared with those in untransfected cells as well as the control siRNA transfected ones ($P<0.05$; Fig. 6C). There were no significant differences in WNV titers between infected cells and those with DMSO treatment or the control siRNA transfection. Collectively, these results demonstrate that the ERK pathway is engaged in controlling WNV propagation in SH-SY5Y cells.

3.7. Role of the ERK pathway in IL-11 induction by WNV infection

The interaction of WNV with the host is key to establish the viral lifecycle and trigger immune responses that lead to neurological and

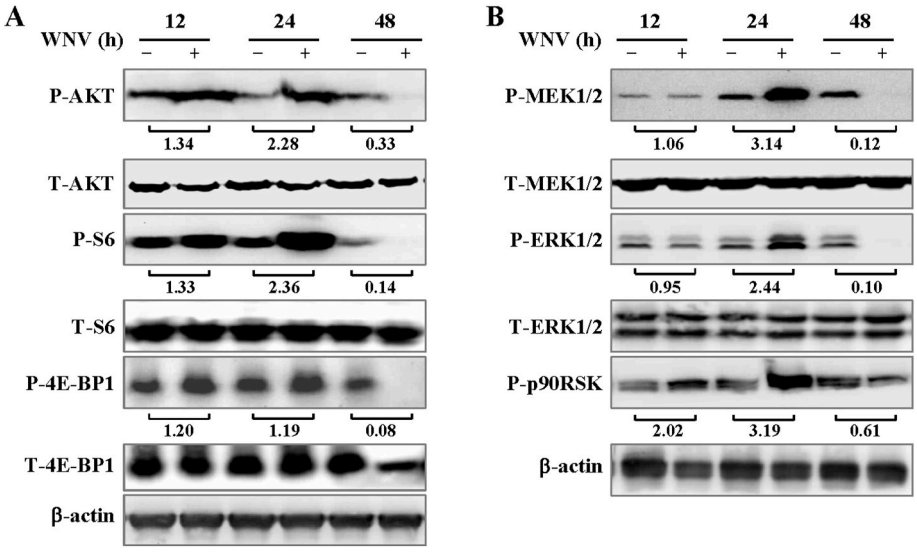

Fig. 4. West Nile virus (WNV) infection regulated AKT and ERK signaling pathways in SH-SY5Y cells. Assessment of AKT (A) and ERK (B) signaling during WNV infection. Cells were incubated with WNV (+) or the mock inoculum (mock; −). Cell lysates were prepared at 12, 24, or 48 h post infection for assessment of total (T-) and phosphorylated (P-) forms of AKT, S6, 4E-BP1, MEK1/2, ERK1/2, or p90RSK by Western blotting. β-actin is shown as a loading control. Fold changes of phosphorylated protein levels in the cells infected with WNV over those in the mock inoculum-incubated cells are shown below each blot after being normalized to β-actin levels. The results are representative of at least three experiments.

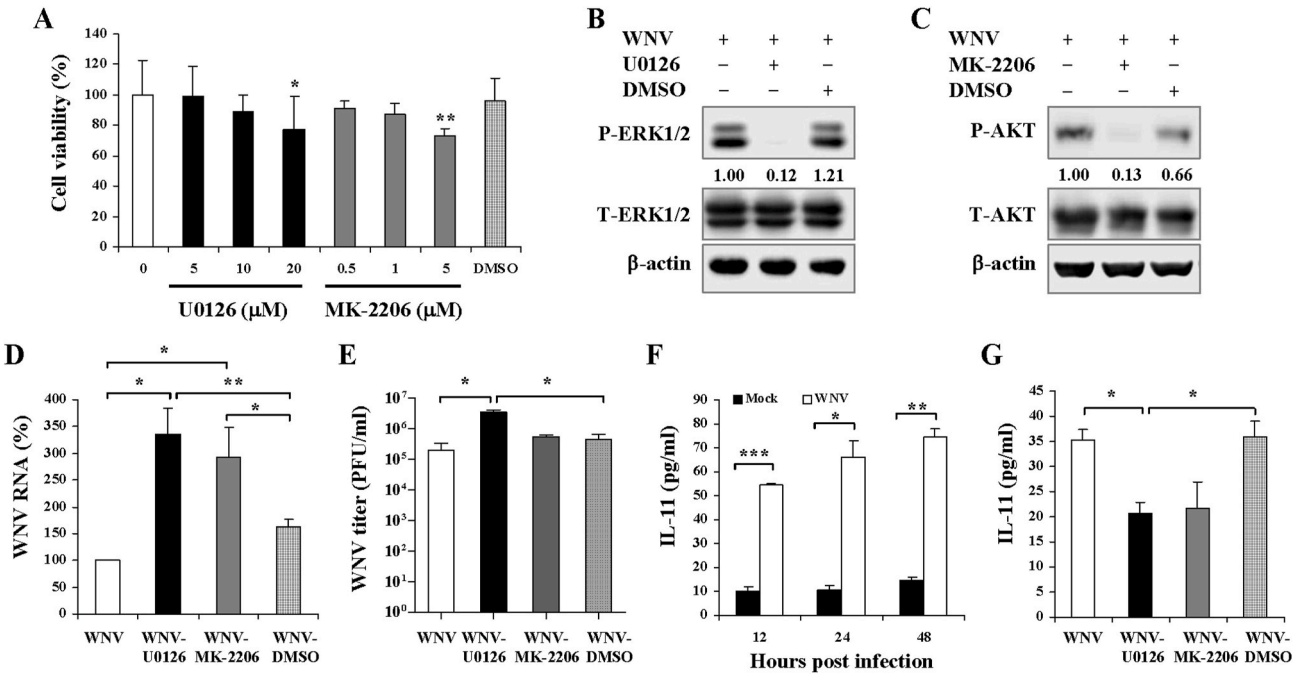

Fig. 5. MK-2206 and U0126 inhibitors influenced West Nile virus (WNV) propagation and IL-11 release in SH-SY5Y cells. (A) Cytotoxicity of U0126 and MK-2206. Cells were treated with U0126, MK-2206, or DMSO dissolvent for 24 h and subjected to cell viability assay. Data are shown as relative percentages of the untreated control (0). *P<0.05, **P<0.005 relative to the untreated control. (B) Inhibition of WNV-mediated ERK1/2 phosphorylation by U0126. (C) Inhibition of WNV-mediated AKT phosphorylation by MK-2206. Cells were pretreated with 10 μM U0126, 1 μM MK-2206, or DMSO and incubated with WNV, followed by treatment with the U0126, MK-2206, or DMSO for 24 h. Western blotting was performed to detect total (T-) and phosphorylated (P-) forms of ERK1/2 and AKT. β-actin is shown as a loading control. Fold changes of phosphorylated ERK1/2 and AKT levels in the cells with the treatment of the inhibitors or DMSO over those in the WNV-infected cells are shown below each blot after being normalized to β-actin levels. One of three representative results is shown. (D) WNV RNA levels upon U0126 or MK-2206 treatment. Real time RT-PCR analysis of WNV RNA levels was performed on extracted RNA from the cells with and without the treatment of U0126, MK-2206, or DMSO. Data are shown as relative percentages of the infected cells. (E) Influence of U0126 and MK-2206 on WNV propagation. In culture supernatants collected from the cells with and without the treatment of U0126, MK-2206, or DMSO, virus titers were determined by plaque assay. (F) IL-11 release induced by WNV infection. Culture supernatants from the cells infected with WNV or incubated with the mock inoculum (mock) were harvested at the indicated time points and IL-11 amounts were measured by ELISA. (G) Effects of U0126 and MK-2206 on IL-11 release induced by WNV. In culture supernatants from the cells with and without the treatment of the inhibitors or DMSO, IL-11 amounts were measured by ELISA. Data are representative of three experiments performed in duplicate. *P<0.05, **P<0.005, ***P<0.001. −, without; +, with.

immunopathological consequences.[26] A lot of pro-inflammatory cytokines initiate severe inflammatory responses during WNV infection. However, modulation of anti-inflammatory cytokines by WNV infection is not well characterized. The IL-6 family cytokines display multiple biological effects and mediate functions in host protective immunity, development of multiple organs and tissue regeneration. IL-11, a member of IL-6 family, has distinct properties that define its unique roles in human disease by acting as a master regulator of fibrosis, tissue integrity, and stromal inflammation.[27] Recently, the roles of IL-11 in inflammatory diseases have been investigated.[28] We have found that mRNA expression of IL-11 was time-dependent up-regulated by WNV (Fig. 2). It was interesting to explore the modulation of the anti-inflammatory cytokine IL-11 by WNV infection and the molecular mechanisms underlying IL-11 modulation by targeting signaling events. Besides mRNA expression, IL-11 release was measured by ELISA in the supernatants from infected SH-SY5Y cells. As shown in Fig. 5F, in comparison with IL-11 amounts in the mock-infected cells, IL-11 amounts were significantly increased in the WNV-infected cells at the indicated time points (for 12 h, P<0.001; for 24 h, P<0.05; for 48 h, P<0.005). Moreover, IL-11 release was enhanced by WNV infection in a time-dependent manner. The induction of IL-11 by WNV was confirmed based on mRNA expression and the release of IL-11.

Whether the ERK and AKT pathways are involved in the IL-11 induction was addressed. Effects of inhibitors on IL-11 induction by WNV were evaluated. ELISA testing showed that IL-11 amounts were higher in infected SH-SY5Y cells than those in mock-incubated cells (35.15 pg/ml vs 11.84 pg/ml). IL-11 amounts were decreased in infected cells with treatment of 10 μM U0126 or 1 μM of MK-2206 (Fig. 5G). In comparison with IL-11 amounts in infected cells as well as cells treated with DMSO, IL-11 amounts were significantly decreased in infected cells treated with 10 μM of U0126 (P<0.05). Such significant decrease was not observed in cells treated with 1 μM of MK-2206. DMSO treatment showed little effects on IL-11 amounts. Accordingly, IL-11 release induced by WNV was down-regulated due to the treatment by inhibitors, with a potent inhibitory effect of U0126.

The role of the ERK pathway in IL-11 induction was further examined in SH-SY5Y cells transfected with the ERK1/2 siRNA prior to WNV infection. Fig. 6A show that levels of total ERK1/2 were significantly down-regulated in cells transfected with the ERK1/2 siRNA as compared with untransfected cells (P<0.005) as well as cells transfected with the control siRNA (P<0.05), suggesting the knockdown of ERK1/2 expression with the specific siRNA transfection. The ERK1/2 phosphorylation induced by WNV was obviously inhibited by the ERK1/2 knockdown. IL-11 amounts were higher in WNV-infected cells than those in the mock-incubated cells (40.34 pg/ml vs 15.79 pg/ml). Fig. 6D show that IL-11 amounts were significantly decreased in infected cells transfected with the ERK1/2 siRNA as compared with untransfected cells as well as cells transfected with the control siRNA (P<0.05), indicating that the ERK1/2 knockdown was responsible for the reduction in IL-11. In addition, the influence of theERK1/2 knockdown on TNF-α expression induced by WNV was also evaluated. TNF-α mRNA expression was markedly increased in cells transfected with the ERK1/2 siRNA (Fig. 6E), implying that the effect of the ERK1/2 knockdown varied among inflammatory cytokines induced by WNV. In accordance with the data obtained from

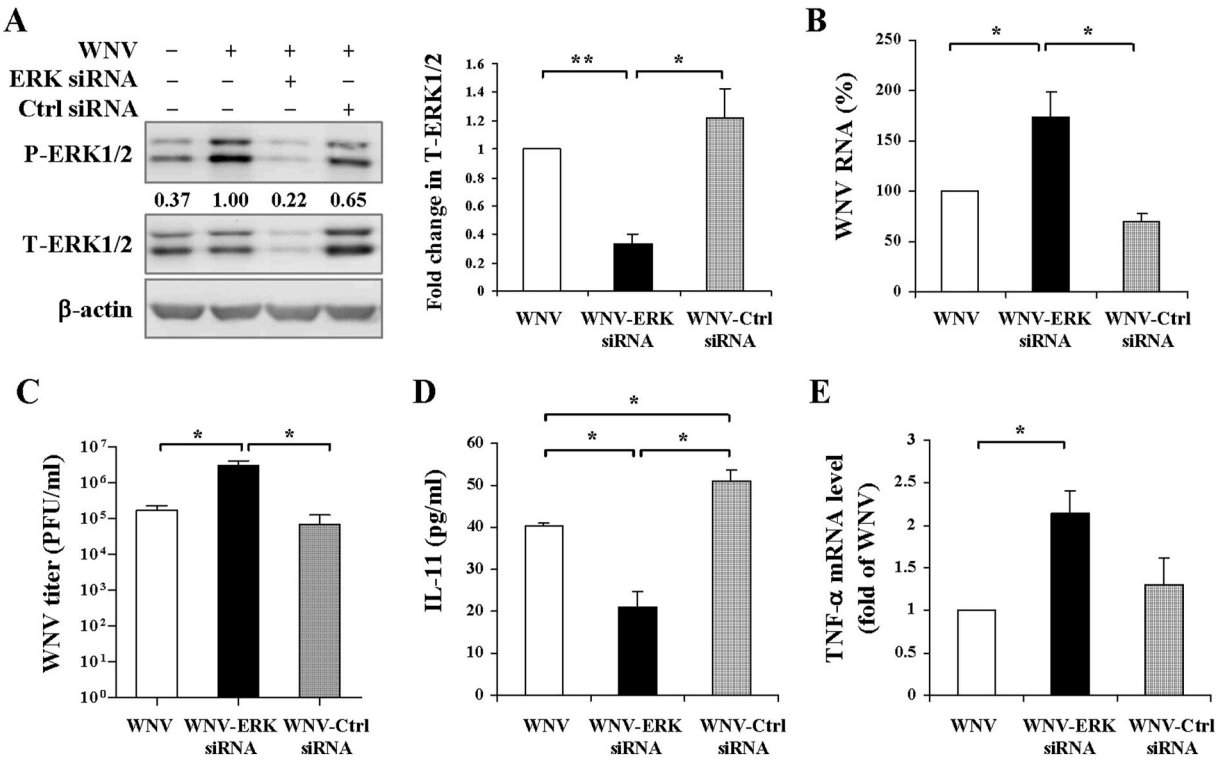

Fig. 6. ERK1/2 knockdown governed West Nile virus (WNV) propagation and IL-11 release in SH-SY5Y cells. (A) Knockdown of ERK1/2 expression. Cells were transfected with ERK1/2 siRNA (ERK siRNA) or control siRNA (Ctrl siRNA). After 72 h transfection, the cells were infected with WNV for 24 h. Total (T-) and phosphorylated (P-) forms of ERK1/2 were detected by Western blotting. β-actin is shown as a loading control. Fold changes of phosphorylated ERK1/2 levels in the cells over those in the WNV-infected cells are shown below each blot after being normalized to β-actin levels. Quantification of T-ERK1/2 expression was made (right panel). Data are expressed as fold changes of T-ERK1/2 levels in the transfected cells over those in the untransfected cells after being normalized to β-actin. (B) WNV RNA levels upon ERK1/2 knockdown. Real time RT-PCR was carried out to determine WNV RNA levels on extracted RNA from the infected cells with and without the siRNA transfection. Data are shown as relative percentages of the infected cells without the siRNA transfection. (C) Influence of ERK1/2 knockdown on WNV propagation. In culture supernatants from the WNV-infected cells with and without the siRNA transfection, virus titers were detected by plaque assay. (D) Influence of ERK1/2 knockdown on IL-11 release. In culture supernatants from the WNV-infected cells with and without the siRNA transfection, IL-11 amounts were measured by ELISA. (E) Influence of ERK1/2 knockdown on TNF-α expression. The mRNA levels of TNF-α were analyzed in the infected cells with and without the siRNA transfection. Data are shown as relative fold of the mRNA levels in the siRNA-transfected cells over those in the untransfected cells. The results are representative of three experiments performed in triplicate. $*P<0.05$, $**P<0.005$. −, without; +, with.

the U0126 treatment, these results showed that the ERK pathway was necessary for IL-11 induction by WNV infection.

4. Discussion

The nfection with WNV is a major cause of mosquito-borne viral neuroinvasive diseases. We have addressed here the regulation of the AKT and ERK signaling pathways by WNV and contributions of such processes to the viral infection and inflammatory response in human neuronal cells, providing new insights into WNV-mediated neuropathogenicity.

Primary cultures of residential cells in human CNS are difficult to obtain and thus used in limited experimental models for WNV infection. Neuronal cell lines are useful models for the elucidation of WNV neuropathology or screening of anti-WNV compounds. For example, polyphenols against WNV were identified in SH-SY5Y cells. WNV-induced inflammation response was evaluated in U251 cells. Neuronal apoptosis caused by WNV was detected in Neuro-2a cells.[29–31] For dissecting WNV tropism in neuronal cells, WNV RNA replication, WNV propagation, and expression of cellular genes involved in inflammatory responses and endoplasmic reticulum stress were determined. Our results show that WNV establishes productive infection in neuronal cell lines of humans and mice, with strong replication efficiency in human SH-SY5Y and U251 cells, indicating that these cells are highly susceptible to WNV infection and support WNV propagation.

During WNV infection, the inflammatory response closely correlates with the development and progression of neuroinvasive disease. Cytokines play a major role in inflammation and immunity against WNV infection. In response to WNV challenge, IL-11, TNF-α, and ATF6 exhibited different expression profiles in a time and cell type-dependent manner. Apart from the multifaceted expression of ATF6, mRNA expression of IL-11 and TNF-α was markedly up-regulated in infected SH-SY5Y and U251 cells. Furthermore, the kinetics of IL-11 and TNF-α mRNA expression was consistent with that of WNV replication in SH-SY5Y and U251 cells, indicating the elicitation of an inflammatory response upon WNV infection. These results may favor the hypothesis that an early weak inflammatory response allows robust WNV replication and that the later strong response is associated with immunopathogenicity, affecting the balance between viral propagation and clearance. In agreement with our results, individuals infected with WNV had abnormally elevated TNF-α.[32] We found that human neuronal cells supported productive WNV replication and WNV elicited a strong inflammatory response. TNF-α has been extensively studied in a variety of model systems in response to viruses. Here we have focused on whether there was a neuronal cell intrinsic role of IL-11 in the acute neuroinflammatory response following WNV infection

The AKT pathway takes part in various cellular processes and is dysregulated in many types of human cancers, and thus considered as a therapeutic target for cancers.[33,34] The WNV capsid protein blocked apoptosis by activating AKT.[35] Our results showed that the signaling cascades of the AKT pathway (AKT-S6-4E-BP1 module) were transiently activated in SH-SY5Y cells upon a short duration of WNV incubation and

moderately activated at 12 h and 24 h post infection, hereas the signaling cascades of the AKT pathway were markedly impaired at 48 h post infection. Dynamic changes in AKT signaling have implications for the status of WNV-infected cells. We propose that the rapid activation of the AKT pathway may facilitate the survival of WNV-infected cells for WNV growth and that the delayed impairment of AKT may be due to cellular damage caused by WNV replication.

The ERK pathway is essential for inter and intra-cellular communication and governs fundamental cellular processes involved in survival, growth, proliferation, metabolism, migration, and differentiation.[36] The dysregulation of the ERK pathway contributes to diseases and is a potential therapeutic target in cancers and neurodegenerative disorders.[37,38] Activation of ERK was induced in mouse embryo fibroblasts, baby hamster kidney cells, and hamster melanoma cells at early time points after WNV infection. Insulin signaling activated Janus kinase-signal transducer and activator of transcription pathway via ERK control WNV infection in mosquito cells.[39,40] We found that the signaling cascades of the ERK pathway (MEK-ERK-p90RSK module) were rapidly and markedly activated in SH-SY5Y cells upon WNV incubation and the strong activation could be detectable at 24 h post infection. Similar to the AKT pathway, the signaling cascades of the ERK pathway were impaired at 48 h post infection. In addition, the regulation of ERK signaling by WNV was also evidenced in Vero cells. For the establishment of infection, WNV activates the ERK and AKT pathways that delay host cell death to extend survival of WNV-infected cells at an early stage. On the other hand, WNV replication in large amounts gives rise to cellular damage that potently impairs ERK and AKT signaling pathways. The distinctive regulation of the AKT and ERK signaling pathways may be implicated in WNV pathogenicity and improve our understanding of the virus-cells interaction controlling WNV infection.

The disruption of the AKT and ERK signal transduction contributes to the pathogenesis of various neurodegenerative diseases.[41] Our results demonstrated that the AKT inhibitor MK-2206 treatment only led to the increase of WNV RNA levels while the MEK1/2 inhibitor U0126 treatment resulted in increased WNV RNA levels and WNV titers. Moreover, both WNV RNA levels and viral titers were significantly increased in SH-SY5Y cells via the knockdown of the ERK1/2 expression. As expected, the ERK1/2 activation was blocked in WNV-infected cells upon the U0126 treatment as well as the knockdown of ERK1/2 expression. Therefore, the inhibition of the ERK signaling triggered by WNV enhanced WNV replication and propagation, implying that the molecular mechanism behind the regulatory aspect of the ERK pathway may provide a new clue to explore WNV pathogenesis.

The importance of cytokines in inflammatory diseases and cancers is being uncovered. IL-11 has important roles in inflammation, fibrosis, and carcinoma, allowing the development of novel therapeutics with the potential for clinical targeting of IL-11 signaling in multiple diseases.[42,43] Studies were performed to define the IL-11 regulation by viruses and its implications for viral pathogenesis. It has been previously demonstrated that up-regulation of IL-11 was observed at 72 h post-infection of cCyprinid herpesvirus.[44] Human cytomegalovirus replication induced IL-11 production.[45] and infection of mice with a lethal dose of H1N1 influenza virus induced IL-11 expression.[46] IL-11 expression at mRNA and protein levels is high during porcine epidemic diarrhea virus infection.[47] IL-11 is elevated at day 9 following BK polyomavirus infection.[48] However, little is known of IL-11 regulation and molecular mechanisms underlying the regulatory effects during WNV infection. Here the manipulation of IL-11 by WNV and association of IL-11 with the ERK pathway were defined. Consistent with the above reports regarding IL-11, our data indicates that WNV infection results in the significant up-regulation of IL-11, in terms of both mRNA expression and secretion. Notably, the U0126 treatment as well as the knockdown of ERK1/2 expression strongly decreased IL-11 production induced by WNV. These results demonstrate that WNV infection potentiates IL-11 production by targeting the ERK pathway, which may contribute to WNV-mediated neuroinflammation. Therefore, the ERK pathway is responsible for controlling WNV propagation and regulating IL-11 induction in human neuronal cells.

In summary, the interaction of WNV and signaling pathways plays a key role in the control of the infection and inflammatory response. Our results suggest that the engagement of the AKT and ERK signaling pathways may be implicated in WNV infection and pathogenesis, helping to understand the mechanisms of WNV survival, propagation, and host inflammatory responses. Further studies are required to comprehensively elucidate the molecular mechanisms of WNV neuropathogenicity based on a variety of experimental models, various virulent strains of WNV, and relevant signaling pathways. The improved understanding of host factors involved in WNV-mediated neuropathology may be essential for facilitating the development of new therapeutic approaches.

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CRediT authorship contribution statement

Wan-Da Tang: Methodology. **Wei-Yang Zhu:** Methodology. **Hai-Lin Tang:** Formal analysis. **Ping Zhao:** Supervision. **Lan-Juan Zhao:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jve.2024.100368.

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