Caspase 3-Dependent Cell Death of Neurons Contributes to the Pathogenesis of West Nile Virus Encephalitis[▽]

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West Nile virus (WNV) is a neurotropic, arthropod-borne flavivirus that has become a significant global cause of viral encephalitis. To examine the mechanisms of WNV-induced neuronal death and the importance of apoptosis in pathogenesis, we evaluated the role of a key apoptotic regulator, caspase 3. WNV infection induced caspase 3 activation and apoptosis in the brains of wild-type mice. Notably, congenic caspase 3^{-/-} mice were more resistant to lethal WNV infection, although there were no significant differences in the tissue viral burdens or the kinetics of viral spread. Instead, decreased neuronal death was observed in the cerebral cortices, brain stems, and cerebella of caspase 3^{-/-} mice. Analogously, primary central nervous system (CNS)-derived neurons demonstrated caspase 3 activation and apoptosis after WNV infection, and treatment with caspase inhibitors or a genetic deficiency in caspase 3 significantly decreased virus-induced death. These studies establish that caspase 3-dependent apoptosis contributes to the pathogenesis of lethal WNV encephalitis and suggest possible novel therapeutic targets to restrict CNS injury.

West Nile virus (WNV) infects and injures diverse neuronal populations in the central nervous system (CNS), leading to encephalitis and death in humans and animals. Human infection is associated with a febrile illness that can progress to a severe neuroinvasive disease characterized by seizures, muscle weakness, cognitive impairment, and a poliomyelitis-like flaccid paralysis (2, 50, 51). WNV induces neuronal damage and loss in several brain regions, including the hippocampus, brain stem, cerebellum, and anterior horn of the spinal cord (13, 21). No therapy or vaccine is currently available for humans, and approximately 10% of individuals with neuroinvasive disease succumb to the infection (36, 40). In addition, long-term neurological sequelae with associated motor, sensory, and cognitive complications are frequent (1, 19, 49). Despite the importance of CNS pathology in severe disease, the mechanisms by which WNV and other encephalitic flaviviruses induce neuronal injury *in vivo* remain largely unknown.

In vitro studies have begun to elucidate the pathways involved in WNV-induced cell death. Caspase 3 is an effector caspase that functions as a central regulator of apoptosis (16, 55). WNV infection triggers apoptosis in different transformed cell lines, resulting in caspase 3 activation, cytochrome *c* release, and exposure of phosphatidylserine on the outer leaflet of the plasma membrane (3, 39). Mouse embryonic stem cell-derived neurons and neuroblastoma cells rapidly undergo apoptosis within 2 to 3 days after WNV infection (39, 53). Several other encephalitic flaviviruses also induce apoptosis: St. Louis encephalitis virus triggered apoptosis in neuroblastoma cells, and Japanese encephalitis virus (JEV) induced apoptosis in cell lines via the endoplasmic reticulum stress pathway (30, 38,

57). These results suggest that induction of programmed cell death may be a common feature of flavivirus replication.

The cellular outcome of WNV replication depends on interactions between host and viral factors. UV-inactivated WNV failed to induce cell death, suggesting that viral replication is required to trigger apoptosis (39). Several WNV proteins may contribute directly to this process. Ectopic expression of the WNV NS3 protein or its helicase or protease domain induced apoptosis and activation of caspase 3 and 8 (44). Expression of WNV capsid protein either *in vitro* or in the striata of mouse brains also triggered apoptosis downstream of caspase 3 and caspase 9 activation (63). Less is known regarding the host factors that modulate cell survival after WNV infection. Phosphatidylinositol 3-kinase signaling is activated by flavivirus infection, which may function to prevent rapid virus-induced death (24). In addition, transcriptional-profiling analysis of WNV-infected cells demonstrated upregulation of selected apoptosis-related genes, including the tumor necrosis factor receptor-associated factor TRAF1, although the physiologic relevance of these observations is unclear (22).

Programmed cell death could have opposing functions during viral infection: it may be antiviral by inducing the death of infected cells, or it may enhance viral spread and progeny release. Cell death can also be pathological if it occurs in nonrenewing cell populations, such as neurons. Given the growing body of evidence that suggests WNV and other flaviviruses induce replication-dependent apoptosis *in vitro*, it has been hypothesized that virus-induced apoptosis may contribute to neuronal death and the pathogenesis of encephalic flaviviruses (39, 45, 60, 63). Indeed, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining in the CNS of WNV-infected mice has been observed (53). However, direct evidence for the mechanism(s) of cell death *in vivo* has been lacking, and the pathways involved in the flavivirus-mediated death of neurons are not well understood. Here, we examined the mechanisms of WNV-induced

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death in primary neurons and the role of caspase 3 in encephalitic disease in mice. We found that caspase 3-dependent cell death *in vivo* contributes to WNV pathogenesis in the CNS, indicating that host cell death pathways may directly promote the pathological manifestations of encephalitis.

MATERIALS AND METHODS

Mice. Wild-type C57BL/6 mice were obtained commercially (Jackson Laboratory, Bar Harbor, ME), and caspase 3^{-/-} mice backcrossed 10 generations onto a C57BL/6 background were obtained from H. Virgin (Washington University, St. Louis, MO). Caspase 3^{-/-} mice on this background develop normally and do not significantly demonstrate neurological abnormalities (25). All mice were genotyped and bred in the animal facility of Washington University School of Medicine, and experiments were approved and performed in accordance with Washington University Animal Studies Committee guidelines.

Mouse experiments and quantitation of viral burden. The WNV strain (3000.0259) was isolated in New York in 2000 and passaged once in C6/36 cells to generate a stock that was used in all experiments. Nine- to 12-week-old mice were used for all *in vivo* studies. Peripheral infection was performed by footpad inoculation of 10⁵ PFU of virus diluted in Hanks balanced salt solution with 1% heat-inactivated (HI) fetal bovine serum (FBS). Intracranial (IC) inoculation was performed with 10¹ PFU of virus diluted in 10 μ l Hanks balanced salt solution with 1% FBS. To determine the kinetics of viral production following peripheral infection, mice were infected by footpad inoculation with 10² PFU of virus, and 5 to 16 mice per time point were euthanized on days 2, 4, 6, 8, and 10. Viral production was also analyzed after IC inoculation with 10¹ PFU of virus on days 2, 4, and 6 after infection of 8 to 12 mice per time point. The mice were perfused with 10 ml PBS, and their organs were removed, weighed, and homogenized. Plaque assays were performed as previously described using BHK21 cells (9).

Immunohistochemistry of brain tissues. To prepare tissues for immunohistochemistry, WNV-infected and uninfected mice were anesthetized with xylazine and ketamine and then intracardially perfused with 10 ml of phosphate-buffered saline (PBS), followed by 10 ml of 4% paraformaldehyde (PFA) in PBS. The tissues were dissected and placed in 4% PFA at 4°C overnight and then cryoprotected in 30% sucrose for generation of frozen sections. TUNEL staining was performed using the NeuroTACS II In Situ Apoptosis Detection kit according to the manufacturer's instructions (Trevigen, Inc., Gaithersburg, MD). Prior to TUNEL staining, tissue sections were rehydrated successively in 100%, 95%, and 70% ethanol, washed with PBS, and digested with proteinase K (1:100 dilution; Trevigen) for 30 min at room temperature. Following quenching with 3% (vol/vol) hydrogen peroxide in methanol, the TUNEL reaction was performed for 1 h at 37°C. WNV antigen was detected by coupling the Catalyzed Signal Amplification and Animal Research Kits (Dako Cytomation, Carpinteria, CA) as previously described (47). Costaining for TUNEL and NeuN was performed by using the In Situ Cell Death Detection Kit according to the manufacturer's instructions (Roche, Indianapolis, IN), followed by blocking and incubation with a monoclonal antibody against the neuron-specific marker NeuN (Chemicon International, Temecula, CA). Sections were then incubated with Cy3-conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody (Invitrogen, Carlsbad, California) and visualized using a Zeiss 510 Meta LSM confocal microscope. Staining for cleaved caspase 3 was performed on 50- μ m floating sections that were quenched with 3% (vol/vol) hydrogen peroxide in methanol for 10 min, washed in PBS, and blocked (2% BSA, 0.2% nonfat dry milk, 0.1% Triton X-100 in PBS) for 1 h at room temperature. The sections were then incubated with an anti-cleaved caspase 3 antibody (Cell Signaling Technologies, Danvers, MA) diluted in blocking buffer overnight at room temperature. Following extensive washing with PBS, the sections were incubated with a biotinylated anti-rabbit secondary antibody for 1 h at room temperature, followed by incubation with avidin-biotinylated enzyme complex reagent according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Staining was developed by incubation with Vector-VIP solution for 5 min (Vector Laboratories), and the tissues were mounted on slides; air dried; dehydrated in 70%, 95%, and 100% ethanol; and visualized.

Caspase activity assay. Caspase 3 and 7 activities were measured in brain tissue using the Caspase-Glo 3/7 kit essentially according to the manufacturer's instructions (Promega, Madison, WI). Nine-week-old C57BL/6 mice were infected by footpad inoculation with 10² PFU of WNV, and on days 2, 4, 6, 8, and 10, animals were perfused with 10 ml PBS and the brains were removed and extracts prepared for use in the Caspase-Glo assay as described previously (31). Briefly, brain extracts were prepared by homogenization in a hypotonic extrac-

tion buffer (25 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM EGTA, and protease inhibitors [Sigma]) and then centrifuged for 12 min at 13,000 rpm. Samples were stored at -80°C, and the protein concentration was normalized prior to use. Plates were read 1 h after reagent addition using a luminometer (Tecan, Durham, NC).

Neuron cultures. Cortical neurons were prepared from embryonic day 15 wild-type and congenic caspase 3^{-/-} mouse embryos essentially as previously described (20). The cells were seeded in 24-well poly-D-lysine/laminin-coated plates at a density of 5 $\times$ 10⁵ cells/well in Dulbecco's modified Eagle's medium containing 5% HI FBS and 5% horse serum. Twenty-four hours after the cells were plated, the neuronal medium was replaced with NeuroBASAL medium supplemented with B27 (Invitrogen) and L-glutamine. Cortical neuron infection experiments were performed using cells cultured for 3 to 4 days. For all *in vitro* experiments, neurons were infected at a multiplicity of infection (MOI) of 0.1 for 1 h at 37°C. Free virus was then removed by serial washing with PBS. For virus production experiments, supernatants were harvested on days 1, 2, 3, and 4 postinfection after being extensively washed 24 h prior to the indicated collection time point. Viral titers were determined by plaque assay on BHK21 cells.

Immunofluorescence of neurons. Cortical neurons were infected with WNV at an MOI of 0.1. Four days later, WNV-infected and control uninfected neurons were fixed with 4% PFA in PBS at 4°C for 15 min. The cells were permeabilized in PBS with 0.2% Triton X-100, blocked (5% normal goat serum and 0.2% Triton X-100), and stained with rabbit antibodies against cleaved caspase 3 (Cell Signaling Technology), the neuron-specific marker MAP-2 (Chemicon International, Temecula, CA), or a control rabbit IgG, and rat anti-WNV immune serum. After being washed, the cells were incubated with Alexa-488-conjugated anti-rabbit IgG (Invitrogen), Cy3-conjugated anti-rat IgG (Jackson Laboratories, West Grove, PA), and the nucleic acid stain TO-PRO-3 (Invitrogen). TUNEL and WNV costaining was performed using the In Situ Cell Death Detection Kit according to the manufacturer's instructions (Roche), followed by blocking and staining with rat anti-WNV immune serum as described above. Neurons were visualized using a Zeiss 510 Meta LSM confocal microscope. The numbers of caspase 3- and WNV-positive cells were counted in images taken of five to seven randomly selected fields from different coverslips harvested from two or three independent experiments.

Western blotting. WNV-infected and uninfected cortical neurons were lysed in RIPA buffer (10 mM Tris, 150 mM NaCl, 0.02% sodium azide, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], pH 7.4), with protease inhibitors (Sigma) on days 1, 2, 3, and 4 after infection. Protein levels were normalized and samples were resolved on a NuPage 12% Bis-Tris gel in MOPS (morpholinepropanesulfonic acid) SDS running buffer (Invitrogen). Following transfer, membranes were incubated in blocking buffer (Tris-buffered saline, 5% nonfat dry milk, 0.1% Tween 20) for 1 h at room temperature. The membranes were probed with caspase 3 and cleaved caspase 3 antibodies (Cell Signaling Technologies) in blocking buffer at 4°C overnight. The blots were incubated with an anti-rabbit peroxidase-conjugated secondary antibody and visualized using LumiGLO Immunoblotting reagent (Amersham Biosciences).

DNA-laddering assay. To detect DNA fragmentation, uninfected cortical neurons and neurons on day 4 after infection at an MOI of 0.1 were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K) and then incubated at 55°C for 60 min. The lysates were centrifuged to pellet cell debris (14,000 $\times$ g for 15 min), and the soluble fraction was treated with RNase A at 55°C for 60 min. The lysates were heated to 70°C, mixed with heated loading buffer, and electrophoresed on a 2% agarose gel.

Neuron survival studies. Cortical neurons were harvested from wild-type and congenic caspase 3^{-/-} embryonic day 15 mouse embryos and seeded at a density of 1 $\times$ 10⁵ cells per well in poly-D-lysine/laminin-coated 96-well plates. For caspase inhibitor studies, wild-type neurons were treated with 50 or 20 μ M of either the pancaspase inhibitor QVD-OPH (MP Biomedicals, Aurora, OH), the caspase 3-specific inhibitor Z-DVED-FMK (Calbiochem, San Diego, CA), or 0.5% dimethyl sulfoxide (DMSO) (vehicle control) for 1 h prior to infection with an MOI of 0.1. The medium was changed on day 2 postinfection, and treated cells were maintained in the presence of the inhibitor or vehicle control throughout the course of the experiment. Neuron survival was assessed on days 1, 2, 3, and 4 after infection using the CellTiter-Blue Cell Viability Assay according to the manufacturer's instructions (Promega). The data were normalized to uninfected neurons harvested at the same time points. To quantitate neuronal survival by TUNEL staining, 5 $\times$ 10⁵ caspase 3^{-/-} and congenic wild-type neurons were seeded onto 12-mm poly-D-lysine/laminin-coated coverslips (BD Biosciences, Bedford, MA). The cells were either left untreated or were treated as described above with 50 μ M QVD-OPH, 50 μ M Z-DVED-FMK, or 0.5% DMSO 1 h prior to infection. On day 4 after infection, the cells were washed with

FIG. 1. Caspase 3 activation and apoptosis in the CNS are triggered by WNV infection. (A) Activated caspase 3 within the CNS of WNV-infected wild-type mice (arrows). Representative images from different areas of the cerebellum and cortex at low (top) and high (bottom) magnification are shown from day 9 after infection after review of three independent brains. The scale bars are equal to 5 μ m unless otherwise noted. (B) Caspase 3/7 activity in the brains of WNV-infected mice. Wild-type mice were infected with 10^2 PFU of WNV by footpad injection, and the brains were harvested on days 2, 4, 6, 8, and 10 after infection. Homogenates were analyzed for caspase 3/7 activity ($n = 5$ to 10 mice per time point). The asterisks represent caspase 3/7 activity levels that were significantly different from those of uninfected mice ($P < 0.05$). The error bars indicate standard errors of the means. RLU, relative light units. (C) TUNEL staining in brains of wild-type mice on day 10 after infection (arrows). Sections were counterstained with hematoxylin, and typical images from the cerebellum and cortex are shown after review of three or four different brains.

PBS and fixed with 4% PFA in PBS for 15 min at 4°C. TUNEL staining was performed with the Fluorescein In Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions, and the cells were counterstained with DAPI. Neurons were visualized using a Zeiss Axiovert 200 microscope. The number of TUNEL-positive cells relative to the total number of cells was determined from five to seven randomly selected fields from coverslips from three or four independent experiments.

Statistical analysis. For in vivo viral-burden analyses, the Mann-Whitney test was used to determine significant differences. Kaplan-Meier survival curves were analyzed by the log rank test, and in vitro experiments with neurons were assessed by an unpaired, two-tailed Student's *t* test. All data were evaluated using Prism software (GraphPad, San Diego, CA).

RESULTS

Caspase 3 is activated in the CNS after WNV infection. To begin to elucidate the molecular basis of WNV-induced neuronal death, brain sections from uninfected and WNV-infected C57BL/6 mice were analyzed for the presence of activated caspase 3. Since activated caspase 3 is labile (59), the brains were examined throughout the infection time course. Activated caspase 3 was detected in cells in multiple regions of the brains of infected mice, including the cerebellum and cortex, at time points (days 8 to 10) corresponding to peak brain viral loads (Fig. 1A and data not shown). To quantitatively examine the kinetics of WNV-induced caspase 3 activation in the CNS, wild-type mice were infected subcutaneously, and brain homogenates were tested on different days by a substrate-specific caspase 3/7 activity assay (Fig. 1B). Caspase 3/7 activity was enhanced beginning on day 6 and remained elevated on days 8

and 10 after infection, correlating with viral entry into the CNS and the onset of encephalitic symptoms and death (9, 33, 48).

To examine whether caspase 3 activation in vivo was associated with the induction of apoptosis, brains from WNV-infected mice were analyzed for detection of TUNEL. Consistent with previous reports (53, 62), TUNEL-positive cells were detected in several areas of the brain, including those that were positive for activated caspase 3 (Fig. 1C). Thus, WNV infection in vivo induces caspase 3 activation and apoptosis in the CNS of infected mice.

Mice deficient in caspase 3 are less susceptible to lethal WNV infection. To investigate the role of caspase 3 in WNV pathogenesis in the CNS in vivo, we evaluated the morbidity and mortality of wild-type and congenic caspase 3-deficient mice after infection with a virulent lineage I North American isolate. Following footpad inoculation with 10^2 PFU, caspase 3^{-/-} and wild-type mice showed similar kinetics of symptom onset, but the overall number of caspase 3^{-/-} mice with overt disease manifestations was lower. Correspondingly, caspase 3^{-/-} mice showed a trend toward higher survival after peripheral WNV infection (80% [$n = 57$] compared to 65% [$n = 92$]) (Fig. 2A). Statistically significant differences were not achieved ($P = 0.06$), however, likely due to the relatively high baseline survival rate of wild-type mice in this infection model. To directly test whether caspase 3 contributes to WNV pathogenesis in the CNS, we inoculated wild-type and caspase 3^{-/-} mice IC with 10^1 PFU and monitored disease symptoms and lethal-

FIG. 2. Caspase 3-deficient mice show resistance to lethal WNV infection. (A and B) Ten- to 11-week-old wild-type (WT) and caspase 3^{-/-} mice were inoculated with WNV by either footpad (A) or intracranial (B) injection and followed for 21 days. The survival curves were constructed using data from two to six independent experiments, where n was 57 and 92 for footpad infection and n was 15 and 60 for intracranial infection of caspase 3^{-/-} and wild-type mice, respectively. (C and D) Viral burden in brain tissues after intracranial WNV infection. WNV burden in the brains of wild-type and caspase 3^{-/-} mice were determined from 5 to 16 samples on the indicated days by plaque assay after footpad (C) or intracranial (D) infection. The dotted lines represent the limit of sensitivity of the assay. The viral burden in caspase 3^{-/-} mice was not statistically different from that in wild-type mice in any tissue samples at any time point ($P \geq 0.3$).

ity. Consistent with previous reports, wild-type mice showed rapid weight loss and severe clinical symptoms by day 6 after infection, including hunching, fur ruffling, impaired balance, and tremors (48, 64). Wild-type mice rapidly succumbed to infection soon after the onset of symptoms, with only a 2% survival rate (Fig. 2B). In contrast, caspase 3^{-/-} mice showed reduced symptoms overall and demonstrated significantly decreased mortality following IC inoculation, with a 33% survival rate ($P < 0.0001$). In addition, the mean time to death of caspase 3^{-/-} mice was significantly extended relative to wild-type mice (8.7 ± 0.9 days versus 7.2 ± 0.8 days, respectively; $P = 0.008$). This phenotype of decreased death after IC WNV infection in a genetically deficient mouse is particularly striking and has not been observed with any other mouse strain (47, 48, 54).

Caspase 3 deficiency does not alter WNV replication and spread. Apoptosis may contribute to the replication and spread of some viruses, such as influenza virus (37, 61). We questioned whether the increased survival rate observed in caspase 3^{-/-} mice was due to a reduction in the WNV burden. To evaluate this, wild-type and congenic caspase 3^{-/-} mice were infected either by footpad or by IC inoculation, and the viral loads were measured over time. Following footpad inoculation, similar levels of infectious virus were detected in the brains of wild-type and caspase 3^{-/-} mice beginning on day 6

after infection ($10^{3.4}$ PFU/g versus $10^{3.1}$, respectively; $P = 0.5$) and peaking on day 8 ($10^{3.6}$ PFU/g versus $10^{3.3}$, respectively; $P = 0.6$), corresponding to the onset of symptoms and death (Fig. 2C). Following IC inoculation, similar amounts of virus were detected in the brains of both mouse strains beginning on day 2 after infection ($10^{2.1}$ PFU/g versus 10^2 for wild-type and caspase 3^{-/-} mice, respectively; $P = 0.9$), with 37% (three of eight) of each strain having viral loads above the limit of detection (Fig. 2D). The viral burdens in the brains of wild-type and caspase 3^{-/-} mice increased on day 4 ($10^{5.7}$ PFU/g versus $10^{5.0}$, respectively; $P = 0.3$) and peaked on day 6 ($10^{7.2}$ PFU/g versus $10^{6.7}$, respectively; $P = 0.5$) after infection, followed rapidly by severe symptoms and the death of the mice. Overall, no significant differences in brain viral loads were detected between wild-type and caspase 3^{-/-} mice at any time point by either inoculation route. Consistent with these observations, the viral burdens were not significantly different in caspase 3^{-/-} and wild-type mice at any time point in all other tissues tested, including the spleen and spinal cord (data not shown) ($P > 0.3$). Thus, the increased survival observed in caspase 3^{-/-} mice was not due to inherent differences in the kinetics of viral dissemination or infection in the CNS.

Caspase 3-deficient mice show decreased neuronal death after WNV infection. As caspase 3 activation correlated with WNV-induced neuronal death in vivo, we hypothesized that

FIG. 3. Caspase 3^{-/-} mice show decreased cell death in distinct neuronal populations. Wild-type and caspase 3^{-/-} mice were inoculated intracranially with WNV, and immunohistochemistry was performed on the brains on day 6 after infection. Characteristic images from infected and uninfected mice are shown after evaluation of three or four independent brains (scale bars = 5 μ m). (A) WNV antigen was detected at high levels in neurons throughout the brain following IC inoculation. TUNEL-stained cells were found in several brain regions in wild-type and caspase 3^{-/-} mice. Arrows indicate cells that are positively stained. (B) Confocal microscopic images of the cerebellum showing that TUNEL staining extensively colocalizes with the neuronal marker NeuN (arrows). An uninfected mouse brain costained for TUNEL and mouse IgG served as a control (scale bars = 5 μ m). (C) The numbers of TUNEL-positive cells in different brain regions were assessed in wild-type and caspase 3^{-/-} mice with comparable levels of WNV antigen staining after review of 10 independent fields per region per mouse. The asterisks indicate values that were statistically different ($P < 0.05$) from those of wild-type mice. The error bars indicate standard errors of the means.

caspase 3^{-/-} mice may survive because of differences in WNV-mediated neuronal death. To examine this directly, WNV antigen and TUNEL staining were performed on parallel brain sections from wild-type and caspase 3^{-/-} mice on day 6 after IC inoculation. As expected, high levels of WNV antigen were detected in the brains of both wild-type and caspase 3^{-/-} mice and localized extensively with neurons in the hippocampus, brain stem, cerebellum, and cortex. TUNEL-positive cells were detected in both groups of mice in brain regions that corresponded to areas of intense WNV antigen staining (Fig. 3A and data not shown). Confocal microscopy costaining experiments with NeuN, a neuron-specific marker (34), and TUNEL confirmed that neurons were the predominant cell population susceptible to WNV-induced cell death (Fig. 3B). To quantify the relative levels of cell death in caspase 3^{-/-} and wild-type mice, brains with comparable WNV antigen staining were examined and the numbers of TUNEL-positive cells were evaluated in different regions. Consistently, brains from caspase 3^{-/-} mice showed reduced numbers of TUNEL-positive cells in the brain stem, cortex, and cerebellum (Fig. 3C; $P \leq 0.03$). Interestingly, TUNEL staining appeared to be equivalent in the hippocampus, suggesting that the requirement for caspase 3 in WNV-induced apoptosis may differ among distinct neuronal populations. These results demonstrate that WNV-infected caspase 3^{-/-} mice show decreased cell death in the context of high viral burdens, suggesting that impairment of caspase 3 activity may be associated with reduced neuronal injury.

WNV infection of primary neurons induces caspase 3 activation. To directly examine the role of caspase 3 activation in WNV-mediated neuronal injury, we isolated primary cortical neurons from wild-type C57BL/6 mice. These cultures were of high purity (>90% for the neuronal marker MAP-2) and exquisitely sensitive to WNV infection (20); costaining experiments confirmed that WNV infection localized to neurons (Fig. 4A). Cytopathic effect in neurons was observed by day 3 after infection, and widespread neuronal damage and death occurred shortly thereafter (data not shown). To evaluate whether WNV-induced death of primary neurons occurred via apoptosis, costaining for WNV antigen and TUNEL was performed. The majority of infected neurons on day 4 after infection were also TUNEL positive (Fig. 4B). Importantly, WNV infection also resulted in the formation of distinctive 180- to 200-bp DNA ladders that are characteristic of endonucleolytic DNA cleavage associated with apoptosis (Fig. 4C). Together, these data suggest that WNV infection induces apoptosis in primary neurons. As apoptosis can occur through biologically distinct caspase-independent and -dependent pathways (23), we examined whether WNV infection also resulted in caspase 3 activation. Primary cortical neurons were costained for WNV antigen and the activated form of caspase 3. Notably, 96% of cells that were caspase 3 positive were also WNV antigen positive on day 4 after infection, although many WNV-positive cells were caspase 3 negative (Fig. 4D). The kinetics of caspase 3 activation after WNV infection was also examined by Western blotting (Fig. 4E). Uninfected neurons displayed uniform

FIG. 4. WNV induces caspase 3 activation and apoptosis in primary neurons. Primary cortical neurons were stained for WNV antigen and costained for the neuronal marker (A) MAP-2, (B) TUNEL, or (D) activated caspase 3. Representative images on day 4 after infection from two or three independent experiments are shown (scale bars = 5 μ m). Cells that show WNV antigen colocalized with the indicated target are indicated (arrows). (C) DNA fragmentation in WNV-infected primary cortical neurons. DNA was extracted from uninfected (uninf) and infected (WNV) neurons on day 4 after infection and resolved by agarose electrophoresis. Std, standard. (E) Western blot of total caspase 3 and cleaved caspase 3 in uninfected and WNV-infected cortical neurons on days 1 to 4 postinfection.

levels of total caspase 3 and low levels of activated caspase 3. In contrast, activated caspase 3 was readily detected in WNV-infected neurons on day 2 and peaked on day 4 after infection. Thus, WNV infection induces significant caspase 3 activation in primary CNS neurons, which corresponds kinetically to the induction of apoptosis and cell death.

WNV-mediated neuronal death is reduced by caspase inhibition. Since both lethality and neuronal death were decreased in mice lacking caspase 3, we questioned whether inhibition of caspase activity could directly improve the survival of neurons after WNV infection. To evaluate this, primary cortical neurons were treated with the pancaspase inhibitor QVD-OPH and monitored using a cell viability assay. Treatment significantly decreased cell death 4 days after WNV infection, with an average 21% reduction compared to vehicle-treated, infected controls (Fig. 5A) ($P \leq 0.002$). To evaluate the contribution of caspase 3 to WNV-mediated death, neurons were treated with the caspase 3-specific inhibitor DEVD-FMK. Notably, caspase 3 inhibition reduced WNV-mediated neuronal death to levels similar to that achieved with the pancaspase inhibitor (Fig. 5B) ($P \leq 0.01$), suggesting that caspase 3 may play a dominant role among caspases in modulating cell death after WNV infection. TUNEL staining was also performed on caspase inhibitor- and vehicle-treated neurons to independently establish that caspase inhibitors blocked WNV-induced cell death. Treatment with the pancaspase and caspase 3 inhibitors reduced TUNEL staining by 18% and 16%, respectively, confirming the ability of caspase inhibitors to increase neuronal survival (Fig. 5C and D) ($P \leq 0.01$). This increase in cell survival was independent of the virus yield, as inhibition of caspase activity had little effect on WNV production over time

(Fig. 5E). To directly test whether WNV-induced neuronal death was modulated by caspase 3, we assessed the survival of cortical neurons derived from caspase 3^{-/-} and congenic wild-type mice. A lack of caspase 3 expression in the deficient neurons was verified by immunohistochemistry and Western blot analysis (Fig. 6A and data not shown). Consistent with our in vivo findings, neurons genetically deficient in caspase 3 demonstrated decreased cell death, with a 23% reduction in the number of TUNEL-positive cells at day 4 after infection compared to the wild type (Fig. 6B) ($P < 0.0001$). The production of infectious virus over time was equivalent in wild-type and caspase 3^{-/-} neurons, similar to results obtained using caspase inhibitors (Fig. 6C). Taken together, caspase inhibition or a genetic deficiency in caspase 3 significantly reduced, but did not completely block, neuronal death. Thus, the WNV-induced death of neurons is partially dependent on the activation of caspase 3.

DISCUSSION

In this study, we demonstrated that caspase 3 activation and apoptosis are associated with WNV-induced neuronal injury and death. Caspase 3^{-/-} mice had increased survival rates after WNV infection, and this phenotype was not associated with changes in viral replication but rather correlated with decreased levels of neuronal death in the brain. Moreover, a genetic deficiency in caspase 3 or pharmacological inhibition of caspase 3 significantly improved the survival of WNV-infected CNS neurons. These experiments demonstrated that caspase 3-dependent cell death directly contributes to the pathogenesis of WNV encephalitis.

FIG. 5. Caspase inhibitors increase neuronal survival after WNV infection. Primary cortical neurons were generated from wild-type mice and treated with vehicle control (black bars) or 50 μ M (gray bars) and 20 μ M (white bars) of a pancaspase inhibitor (A) or a caspase 3-specific inhibitor (B). The neurons were infected at an MOI of 0.1, and survival was evaluated by a fluorescence-based viability assay on days 1, 2, 3, and 4 after infection. The data represent the averages from 4 or 5 independent experiments. The error bars indicate standard errors of the mean. (C) Representative images of TUNEL (green) and DAPI (blue) nuclear staining in vehicle- and pancaspase inhibitor-treated cortical neurons (scale bar = 50 μ m). D4 p.i., day 4 postinfection. (D) The percentages of TUNEL-positive cells after vehicle and caspase inhibitor treatment were quantitated by immunofluorescence microscopy on day 4 after infection. DAPI was used as a nuclear counterstain to determine total cell numbers. The data represent the averages of three or four independent experiments. (E) Daily infectious-virus production was determined following treatment with vehicle control or 20 μ M and 50 μ M of the pancaspase inhibitor. The data reflect the averages from three independent experiments. The asterisks indicate differences that were statistically significant ($P < 0.05$).

Following entry into the CNS, WNV replicates in neurons and induces injury and death that results in severe neurological symptoms. Our experiments established that caspase 3 activation correlates with the induction of apoptosis in neurons. These results are consistent with *in vitro* studies in nonneuronal transformed cells that demonstrated that WNV infection triggers apoptosis (3, 39). Caspase activation and apoptosis appear to be features of flavivirus infection of cells. JEV initiated caspase 3 activation and apoptosis in immortalized cells (24, 29, 30), and dengue virus-induced apoptosis has been documented in multiple cell types *in vivo* and *in vitro*, including liver, brain, and epithelial cells (5, 6, 8, 15).

Caspase 3-dependent apoptotic cell death of WNV-infected neurons could be a protective or pathological host response. Apoptosis can act as an innate defense that restricts viral spread by eliminating infected cells and triggering pathogen recognition pathways (58). Alternatively, cell death could directly contribute to the spread and replication of WNV. Indeed, caspase 3 activation promotes the release of influenza

virus RNA-protein complexes from the nucleus (61), and mice deficient in caspase 3 show blunted reovirus replication, myocardial injury, and lethality (7). Additionally, mice infected with Sindbis virus expressing the antiapoptotic protein Bcl-2 had decreased lethality that was associated with reduced apoptosis and viral replication (26). A lack of caspase 3 activity, however, did not alter the kinetics of WNV replication or spread in neurons *in vitro* or *in vivo*. Analogously, inhibition of cell death by Bcl-2 overexpression did not alter the spread or production of JEV (30). The contribution of caspase 3-mediated cell death to viral replication thus varies among viral families and may reflect fundamental differences in viral replication and secretion strategies. Our experiments are consistent with a model in which the possible protective effects of caspase 3-dependent apoptosis in WNV encephalitis are offset by an excessive loss of nonrenewable neurons in the CNS.

Caspases play important roles in the initiation and execution of apoptosis (46) but also have additional nonapoptotic functions, including cytokine maturation (caspases 1, 4, and 5) (10,

FIG. 6. A deficiency in caspase 3 enhances neuronal survival after WNV infection. Primary cortical neurons were isolated from caspase 3^{-/-} and congenic wild-type mice and infected at an MOI of 0.1. (A) Neurons were costained for WNV antigen and activated caspase 3 (scale bars = 5 μ m). The arrow indicates a double-positive cell. (B) The percentages of TUNEL-positive cells were determined by immunofluorescence microscopy on day 4 after infection. The values were obtained by quantitating the total cell number using a nuclear counterstain (DAPI) and the number of TUNEL-positive cells. The data represent the averages from three independent experiments. The error bars indicate standard errors of the means. WT, wild type. (C) The production of infectious virus by wild-type and caspase 3^{-/-} neurons in 24-h intervals was determined by plaque assay. The asterisks indicate values that were statistically different from those of wild-type mice ($P < 0.05$).

12, 32), T-cell activation (caspase 8) (4), and monocyte differentiation (caspase 8) (17, 56). Given the multiple functions of caspases, we directly tested whether the increased survival of caspase 3^{-/-} mice after WNV infection was due to alterations in cell death. Importantly, decreased numbers of TUNEL-positive cells were observed in the brains of equivalently infected caspase 3^{-/-} mice compared to wild-type mice. Based on this, we suggest that caspase 3 contributes to WNV patho-

genesis by promoting the death of infected neurons in the CNS. However, a genetic deficiency of caspase 3 did not protect all CNS neurons equivalently, as the death of hippocampal neurons after WNV infection appeared largely independent of caspase 3. Analogously, different neuronal subtypes in vitro have distinct survival kinetics after WNV infection. CNS-derived cortical neurons largely die by day 4 after infection, whereas peripheral sympathetic motor neurons survive for 1 to 2 weeks after infection despite producing equivalent amounts of virus (47). Elegant studies have shown that Sindbis virus infection triggers distinct neuronal-death pathways that vary according to the subtype and maturation state of the neurons (11). Mortality in Sindbis virus-infected neonatal mice was reduced by overexpression of cell death regulatory proteins, including Bcl-2, Bax, CrmA, and beclin-1 (26–28, 35), whereas Bcl-2 and Bax expression in adult mice protected hippocampal but not spinal cord neurons from virus-induced death (18). Although further study is warranted, we speculate that different neurons may undergo cell-specific death programs in response to WNV infection.

Flaviviruses can induce cell death directly through viral replication and the production of proapoptotic proteins (41, 43, 44, 52, 57). Notably, pancaspase and caspase 3 inhibitors reduced the WNV-mediated death of primary neurons, which is consistent with studies showing that caspase inhibitors reduced apoptosis after transgenic expression of the flavivirus NS3 and E genes (41, 42, 44). Caspase-dependent apoptosis of neurons in the CNS may be a general mechanism by which neuroinvasive flaviviruses induce injury. However, a deficiency in caspase 3 did not completely protect neurons in vitro or in vivo from WNV-mediated death. Caspase-independent cell death pathways likely also contribute to WNV pathogenesis, potentially through the induction of apoptosis or necrosis. For example, in the absence of functional caspase 3, WNV infection could trigger apoptosis through the activation of noncaspase proteases, such as calpain and cathepsin family proteins (14, 23). In addition, WNV can induce necrosis in vitro in cells exposed to very high viral inocula (3). While it is clear that caspase 3-dependent cell death contributes to lethal WNV infection, future studies are necessary to define the relative contributions of other cell death pathways to WNV pathogenesis. Given the importance of cell death in WNV encephalitis, novel inhibitors that target caspases or other proteases and penetrate the blood-brain barrier could be effective therapeutics for mitigating disease caused by encephalitic flaviviruses. A more complete understanding of the viral and host molecules that result in the death of essential neurons will likely promote the development of novel targets for ameliorating acute and long-term neurological complications of CNS viral infections.

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