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CXCR3 Mediates Region-Specific Antiviral T Cell Trafficking within the Central Nervous System during West Nile Virus Encephalitis¹

Bo Zhang,* Ying Kai Chan,* Bao Lu,[‡] Michael S. Diamond,*^{†§} and Robyn S. Klein^{2*†¶}

Regional differences in inflammation during viral infections of the CNS suggest viruses differentially induce patterns of chemoattractant expression, depending on their cellular targets. Previous studies have shown that expression of the chemokine CXCL10 by West Nile virus (WNV)-infected neurons is essential for the recruitment of CD8 T cells for the purpose of viral clearance within the CNS. In the current study we used mice deficient for the CXCL10 receptor, CXCR3, to evaluate its role in leukocyte-mediated viral clearance of WNV infection within various CNS compartments. WNV-infected CXCR3-deficient mice exhibited significantly enhanced mortality compared with wild-type controls. Immunologic and virologic analyses revealed that CXCR3 was dispensable for control of viral infection in the periphery and in most CNS compartments but, surprisingly, was required for CD8 T cell-mediated antiviral responses specifically within the cerebellum. WNV-specific, CXCR3-expressing T cells preferentially migrated into the cerebellum, and WNV-infected cerebellar granule cell neurons expressed higher levels of CXCL10 compared with similarly infected cortical neurons. These results indicate that WNV differentially induces CXCL10 within neuronal populations and suggest a novel model for nonredundancy in chemokine-mediated inflammation among CNS compartments. *The Journal of Immunology*, 2008, 180: 2641–2649.

The infiltration of virus-specific CD8 T cells is essential for clearance of many viral infections within the CNS (1, 2). Loss of T cells or their effector functions through genetic deletions results in fatal or persistent CNS viral infections, depending on the viral etiology (3–7). Because virus-specific T cells resolve viral infections via cytotoxic mechanisms as well as those that are cell-sparing (8, 9), their recruitment into the CNS, a tissue site with little reserve for cellular loss, must be tightly regulated. Additionally, as the various CNS regions display heterogeneity in the types of neuronal and non-neuronal cells, leukocytes likely traffic in response to a variety of nonredundant, molecular cues that differ between the CNS compartments. Indeed, the patterns of inflammatory infiltrates that occur during viral infections of the CNS vary with the etiology of infection (10–13), suggesting that there are virus- and/or target cell-mediated, regional differences in the recruitment of mononuclear cells that enter the CNS for the purpose of viral clearance.

Chemokines are a family of chemoattractant molecules that direct the recruitment of virus-specific T cells into the CNS after infection (14). In humans and other vertebrate animals, activated T

cells that infiltrate the CNS during encephalitic DNA or RNA viral infections up-regulate expression of the chemokine receptors CCR1, CCR2, CCR5, and CXCR3 and their chemokine ligands CCL2 (CCR2), CCL3 (CCR1, CCR5), CCL4 (CCR5), CCL5 (CCR1, CCR5), and CXCL9–CXCL11 (CXCR3) (15–24). Although targeted deletion of CXCR3 or CXCL10 is associated with a reduction in infiltrating mononuclear cells and enhanced mortality from infection of the CNS with mouse hepatitis virus (MHV)³ (25, 26), few studies have examined regional differences in the expression of chemokine ligands during viral encephalitis. Intranasal infection of an attenuated rabies virus resulted in CCL3 levels that were higher in the cortex, and CCL2, CCL4, and CXCL10 levels that were higher in the cerebellum (27). This was associated with higher levels of CD8 mRNAs in the cortex and higher levels of CD4, CD11b, and CD19 mRNAs in the cerebellum, suggesting that region-specific patterns of chemokine expression may direct the differential trafficking of leukocyte subsets.

West Nile virus (WNV) is a neurotropic flavivirus now endemic within the Northern hemisphere that cycles between ornithophilic mosquitoes and natural bird reservoirs but may also incidentally infect humans and other vertebrate animals (28–30). Although most WNV infections are asymptomatic or manifest as a mild, flu-like illness, potentially fatal neuroinvasive infections occur in the elderly or those who are immunocompromised, including meningitis, encephalitis, and anterior myelitis. In the CNS, WNV targets cortical, midbrain, cerebellar, and spinal cord neurons leading to their injury or death (31–34). The high incidence of WNV neuroinvasive disease in patients on anti-T cell therapies (35, 36) and in mice with T cell deficiencies (37–40) indicate that, similar to other neurotropic viruses, the clearance of WNV within the CNS relies heavily on cell-mediated immune responses that promote the

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³ Abbreviations used in this paper: MHV, mouse hepatitis virus; LCMV, lymphocytic choriomeningitis virus; WNV, West Nile virus.

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migration and effector functions of T cells into the CNS parenchyma. In several of these studies, severe cerebellar involvement was associated with worst outcomes, including fatality (10, 41, 42). Thus, T cell-mediated immunity appears to be particularly important for control of WNV within this CNS region.

Experiments in mice have established that some chemokines and their receptors have essential roles in directing leukocytes to the CNS to clear WNV from infected neuronal cells. CCL3–CCL5, chemokines that all bind the chemokine receptor CCR5, are strongly induced in the CNS after WNV infection (43–46), and targeted deletion of CCR5 is associated with depressed leukocyte trafficking, increased viral burden, and enhanced mortality (43). WNV encephalitis is associated with the early expression of CXCL10 by virally infected neurons that proceeds in a caudal-to-rostral direction within the CNS with significantly higher levels detected in the cerebellum by day 5 postinfection (44). Loss of CXCL10 was associated with decreased recruitment of WNV-specific CD8 T cells into the CNS, high CNS viral loads, and enhanced mortality. Given the observed nonredundant roles for chemokines during WNV encephalitis and the initial observation that CXCL10 exhibits regional variation in its expression by WNV-infected neurons, we determined the contribution of CXCR3 and its ligands, CXCL9 and CXCL10, in antiviral immune responses within different CNS compartments during WNV infection. We report that CXCR3 and CXCL10 are required for T cell-mediated clearance of WNV primarily within the cerebellum during WNV encephalitis. T cell trafficking and effector functions were significantly diminished within the cerebellar compartment compared with other CNS regions in CXCR3-deficient mice. This regional heterogeneity in T cell effector function was associated with the differential up-regulation of CXCL10 by WNV-infected cortical vs cerebellar neurons. These data are the first to indicate that chemokine receptors may control viral burden within the CNS in a region-specific manner.

Materials and Methods

Animals

Five- or 8-wk-old male and female C57BL/6J (*H-2K^bD^b*) mice (Jackson Laboratories) and their backcrossed (six to seven generations) CXCL10^{-/-} (44), CXCR3^{-/-} (47) and CXCL9^{-/-} (48) (courtesy of J. Farber, Bethesda, MD) counterparts were used for all studies. The CXCL10^{-/-} and CXCR3^{-/-} mice were bred at the Washington University School of Medicine, and all studies were performed in compliance with the guidelines of the Washington University School of Medicine Animal Safety Committee.

Mouse model of WNV infection

The WNV strain 3000.0259 was isolated in New York in 2000, passaged once in C6/36 *Aedes albopictus* insect cells, and was used for all studies (49). BHK21 cells were cultured as previously described (50). Five- or 8-wk-old age-matched mice were inoculated subcutaneously via footpad injection with 10² PFU of WNV, which was diluted in HBSS and 1% heat-inactivated FBS. For immunohistochemical analyses, CNS tissues were harvested after perfusion with PBS and 4% paraformaldehyde, incubated in 4% paraformaldehyde for 24 h at 4°C, frozen in cryomedium, sectioned, and stained. For virologic analysis, tissues were weighed, homogenized using a bead-beater, and quantitated by viral plaque assay as previously described (50).

Antibodies

Rat polyclonal Abs against WNV Ag have been described in detail elsewhere (51), and unconjugated rabbit polyclonal Abs against CXCR3 (Zymed Laboratories), BrdU (Roche), and Alexa Fluor-conjugated secondary Abs (Invitrogen: Molecular Probes) were purchased commercially. For flow cytometry, fluorescently conjugated Abs against CD3, CD4, CD8, NK1.1, and IFN- γ were purchased from BD Pharmingen.

Virus infection of neuronal cultures

Primary cultures of purified granule cell neurons were prepared as previously described (52). Purification via Percoll step-gradient centrifugation yields cultures containing ~97% granule cells and ~3% Purkinje neurons (52). Cortical neurons were prepared from embryonic day 15 (E15) mouse embryos as previously described (44). Purity of cortical cultures was determined via staining with anti-MAP-2 (Sigma-Aldrich) and anti-GFAP (Dako) Abs (~95%). All experiments were performed on neurons cultured for 4–6 days. Primary neurons were infected over a range of virus concentrations. After a 1-h incubation at 37°C, free virus was removed by serial washing with neuron medium, and cells were incubated for an additional 24 h. Supernatants were harvested for viral plaque assay, and cells were collected for purification of cellular RNA. The production of infectious virus was measured by plaque assays on BHK21 cells as previously described (50). The cellular RNA from infected neurons was harvested and purified using the RNeasy kit according to the manufacturer's instructions (Qiagen).

Lymphocyte isolation from CNS

Brain-infiltrating T cells were isolated and quantified as previously described (44). In brief, CNS tissues (frontal cortices and cerebella) from WNV-infected, wild-type, and CXCR3^{-/-} mice were dissected from PBS-perfused (20 ml) mice on days 8 or 9 postinfection and dispersed into single-cell suspension in RPMI with 10% FCS. Brain tissues were then harvested and dispersed into single-cell suspension in RPMI 1640 with 10% FCS, 1% glutamine, and 1% PenStrep (Invitrogen). Cells were washed in RPMI and digested with 0.05% collagenase D, 0.1 μ g/ml trypsin inhibitor TLCK, 10 μ g/ml DNase I (all from Sigma-Aldrich), and 10 mM of HEPES (Life Technologies) in HBSS for 1 h. Viable cells were separated by Percoll gradient (70/37/30%) centrifugation for 30 min. Cells were placed in blocking solution containing PBS/1% BSA and stained for CXCR3, CD3, CD4, and CD8. Stained cells were fixed with 2% paraformaldehyde in PBS at 4°C. Data collection and analysis were done using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

Intracellular IFN- γ staining

Intracellular IFN- γ staining of wild-type, CXCR3-, and CXCL10-deficient splenocytes or CNS-derived leukocytes was performed as described (53). Briefly, at day 7 after infection, erythrocyte-depleted splenocytes from WNV-infected wild-type, CXCL10^{-/-}, and CXCR3^{-/-} mice were stimulated at a concentration of 10⁶/100 μ l with 0.1 μ g/ml of either an immunodominant D^b-restricted NS4B WNV peptide or an irrelevant WNV peptide for 4 h at 37°C with the addition of Golgi plug (1 μ l/ml; BD Biosciences). In parallel experiments, CNS-derived leukocytes were harvested from WNV-infected wild-type or CXCR3^{-/-} mice as described above and then stimulated in a similar fashion with WNV peptide. Cells were then incubated with FITC-conjugated CD8 or an isotype FITC-conjugated Ab (BD Biosciences) for 30 min at 4°C. Subsequently, cells were washed and fixed in 2% paraformaldehyde, permeabilized with 0.5% saponin, and stained with an allophycocyanin-conjugated anti-IFN- γ Ab or allophycocyanin-conjugated isotype control. After final washing, staining was assessed by flow cytometry, and the percentage of CD8⁺ lymphocytes that expressed IFN- γ was determined by flow cytometry.

Immunohistochemistry

Frozen sections were washed with PBS and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich), and nonspecific Ab was blocked with 10% normal goat serum (Santa Cruz Biotechnology) for 1 h at room temperature. Monoclonal or polyclonal Abs specific for WNV Ag, CD3, CD31, or BrdU were applied at 1–10 μ g/ml in PBS containing 10% goat serum and 0.1% Triton X-100 overnight at 4°C. Primary Abs were detected with secondary goat anti-rabbit or mouse IgG conjugated to Alexa Fluor 555 or Alexa Fluor 488 (Invitrogen: Molecular Probes) for immunofluorescence or biotinylated for chromagen staining (Dako). In the former case, nuclei were counterstained with DAPI; in the latter case, sections were incubated with streptavidin-conjugated HRP, diaminobenzidine, and counterstained with hematoxylin.

Adoptive transfer studies

WNV-specific CD8 T cells were derived from the spleens of WNV-infected C57BL/6 mice 7 days after infection via negative selection using magnetic beads according to the manufacturer's protocol (Miltenyi Biotec). Naive CD8 T cells were isolated in a similar fashion from the spleens of uninfected C57BL/6 mice. Recipient CXCR3^{-/-}, WNV-infected mice each received 2.5 \times 10⁶ naive or WNV-primed CD8 T cells on day 5

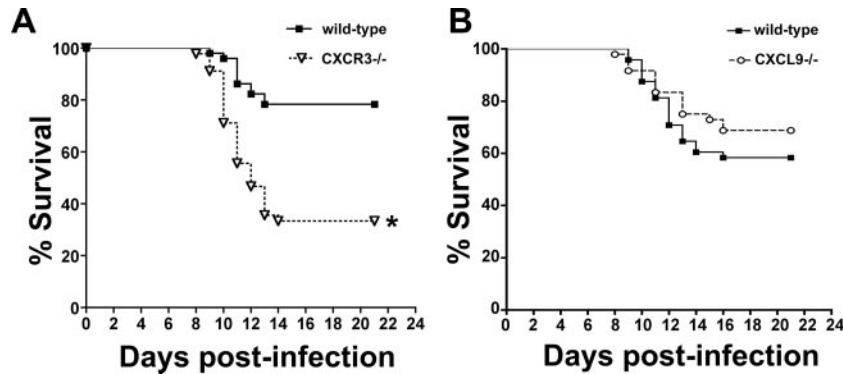


FIGURE 1. Survival data of wild-type and CXCR3^{-/-} and CXCL9^{-/-} mice. Eight-week-old mice were inoculated via footpad with 10² PFU of WNV and followed for 22 days (A). The survival curves were constructed using data from three independent experiments with $n = 12$ –15 mice for each group. Survival differences between wild-type and CXCR3-deficient mice were statistically significant ($p = 0.0001$). Eight-week-old wild-type and congenic CXCL9-deficient C57BL/6 mice were inoculated via footpad with 10² PFU of WNV and followed for 25 days (B). The survival curves were constructed using data from two independent experiments with $n = 10$ for each group. Survival differences and survival times between wild-type and CXCL9-deficient mice were not statistically significant ($p = 0.12$).

postinfection, a time point at which WNV is first detectable within CNS tissues (50).

Real-time quantitative RT-PCR

Total RNA was prepared from WNV-infected tissues or cultured neurons as previously described (44). Following DNase I treatment (Invitrogen), RiboGreen (Invitrogen: Molecular Probes) was used to quantitate total RNA, and cDNA was synthesized using random hexamers, oligodT15, and MultiScribe reverse transcriptase (Applied Biosystems). A single reverse transcription master mix was used to reverse transcribe all samples in order to minimize differences in reverse transcription efficiency. The following conditions were used for reverse transcription: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. All oligonucleotide primers used for quantitative PCR were designed using Primer Express v2.0 (Applied Biosystems). CXCL10 mRNA levels were detected via quantitative RT-PCR using previously published forward and reverse primer sets and protocols (44). Calculated copies were normalized against copies of the housekeeping gene GAPDH.

Statistical analysis

All values are expressed as mean \pm SEM. Survival analysis was performed using the log-rank test. Statistical significance of viral burden was performed using the nonparametric Wilcoxon rank-sum (Mann-Whitney U) test or Student t test with values of $p < 0.05$ considered to be statistically significant.

Results

Loss of CXCR3, but not CXCL9, leads to enhanced mortality from WNV encephalitis

In previous studies, targeted deletion of CXCL10 led to a significant decrease in survival of 8-wk-old mice infected with WNV, with only 10% of CXCL10^{-/-} mice surviving compared with 80% of wild-type controls. The CXCL10 receptor, CXCR3, normally binds two other chemokine ligands, CXCL9 and CXCL11. In the current study, we evaluated the role of CXCR3 and its additional ligands in the T cell-mediated clearance of WNV within the CNS. However, in C57BL/6 mice, the CXCL11 gene has undergone a frame-shift error leading to loss of protein expression, whereas in 129/Sv it has not (National Center for Biotechnology Information Mouse Genomic Sequence Database, accession nos. NT_109320, NW_001030791, NT_039339). Given that our model uses the C57BL/6 strain, we limited our *in vivo* analyses to CXCR3 and its two remaining ligands, CXCL9 and CXCL10.

To define the *in vivo* role of CXCR3 expression during WNV encephalitis, we examined survival and CNS infection in animals that had a genetic deficiency of CXCR3. Eight-week-old CXCR3^{-/-} and congenic wild-type C57BL/6 mice were inocu-

lated with 10² PFU of WNV and followed for survival. Targeted deletion of CXCR3 led to a 50% increase in mortality after WNV infection (Fig. 1A, $p = 0.0001$). In contrast, WNV infection of genetically CXCL9^{-/-} congenic C57BL/6 mice did not lead to a significant alteration in the overall survival rate or average survival time of either 8-wk-old (Fig. 1B) or 5-wk-old mice (data not shown). These data suggest that CXCL10, but not CXCL9, is the CXCR3 ligand required for recovery from WNV encephalitis.

CXCR3 is required for control of WNV infection within the cerebellum

To further evaluate the role of CXCR3 in clearance of WNV from various tissues, we performed virologic examination of spleen and several CNS regions from WNV-infected CXCR3-deficient and wild-type mice at days 2, 5, and 8 postinfection. CXCR3-deficient mice displayed the same kinetics of infection as did wild-type animals, with viral loads detectable at day 5 postinfection within the spleen and at day 8 postinfection within CNS tissues (Fig. 2). However, while loss of CXCR3 did not significantly affect clearance of WNV within the spleen or the level of viral burden in the spinal cord or frontal cortex (Fig. 2A–C), CXCR3^{-/-} mice displayed a 3–4 log-fold increase ($p = 0.02$) in detectable virus in the cerebellum compared with similarly infected wild-type mice (Fig. 2D). In separate studies, we examined IFN- γ production after *ex vivo* stimulation of splenocytes with an immunodominant D^b-restricted WNV peptide against the NS4B protein (53) and did not detect any decrease in the numbers of IFN- γ -producing CD8⁺ T cells in CXCR3^{-/-} or CXCL10^{-/-} mice (data not shown). Additionally, evaluation of the total numbers of CD4⁺, CD8⁺, and CD11b⁺ cells within the spleens and blood of WNV-infected wild-type, CXCR3^{-/-}, and CXCL10^{-/-} mice at days 5 and 7 postinfection revealed no overall differences in the numbers of mononuclear cells (data not shown), suggesting that loss of these molecules did not grossly impact the trafficking patterns of cells involved in the evolution of adaptive immune responses. Taken together, these data suggest that CXCR3 is dispensable for control of WNV infection in the periphery but is specifically required for control within the cerebellar compartment.

Previous studies observed that WNV Ag within the cerebella of wild-type mice with WNV encephalitis is primarily detected within scattered Purkinje neurons and few granule cell neurons (33, 34, 54). Given the high viral loads observed within the cerebella of CXCR3^{-/-} mice, we speculated that additional neuronal

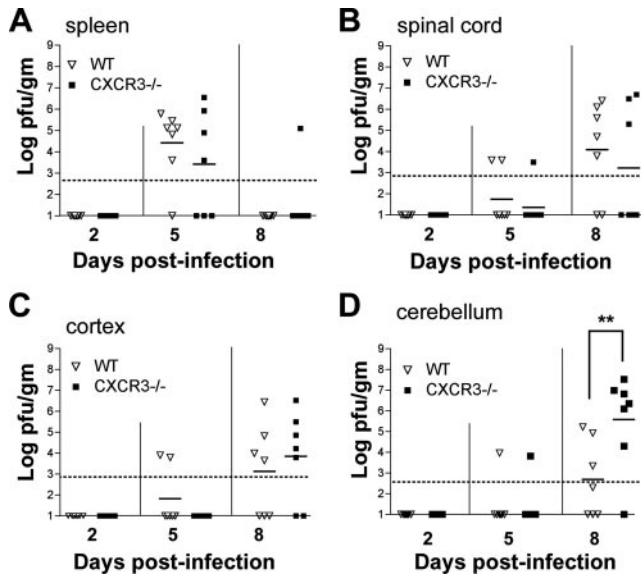


FIGURE 2. Viral loads in the spleen and CNS after WNV infection. WNV burden in the (A) spleens, (B) spinal cords, (C) frontal cortices, and (D) cerebella of wild-type and CXCR3-deficient mice at days 2, 5, and 8 after infection. Virus levels were measured using a plaque assay after tissues were harvested. Data are shown as the average PFU per gram of tissue and reflect 7–10 mice per time point per group. The dotted line indicates the limit of sensitivity of the assay. Horizontal bars indicate the arithmetic mean of the log-transformed data. Asterisks indicate time points at which differences were statistically significant ($p < 0.05$).

cell types could be infected in these animals. Immunohistochemical staining for WNV Ag detected similar scattered infection of neurons within the frontal cortices of wild-type, CXCR3^{-/-}, and CXCL10^{-/-} mice (Fig. 3A–C). In contrast, similar analyses of cerebellar tissues revealed a prominent extension of WNV infection to granule cell neurons in mice with deletion of CXCR3 or CXCL10 (Fig. 3D–F). Control tissues from uninfected mice revealed no staining for WNV Ag (data not shown). These data suggest that loss of CXCR3 or CXCL10 leads to an increase in the range and numbers of cerebellar neurons targeted by WNV during CNS infection.

CXCR3-expressing, CD8⁺ T cells show increased trafficking to the cerebellum

To elucidate the mechanism for CXCR3-dependent control of WNV infection within the cerebellum, we evaluated the numbers of CXCR3-expressing T cells that trafficked into the frontal cortices and cerebella of mice with WNV encephalitis via flow cytometry (Fig. 4). Leukocytes were isolated from individual CNS regions from WNV-infected, wild-type mice at day 9 postinfection and examined for expression of CD3, CD4, CD8, and CXCR3. Although the percentages of CD4⁺ and CD8⁺ T cells were similar between the two regions (Fig. 4A), the percentages of CXCR3-expressing T cells were significantly higher in the cerebellum (Fig. 4B). Examination of IFN- γ production after ex vivo stimulation of leukocytes derived from the frontal cortices and cerebella of WNV-infected mice with our immunodominant D^b-restricted WNV peptide (53) revealed increased percentages of CD8⁺ T cells expressing IFN- γ within the cerebellum that expressed significantly higher levels of CXCR3 (Fig. 4C). These data are consistent with the observed lack of control of viral infection within the cerebella of CXCR3-deficient mice. Levels of several chemokine receptors expressed by activated T cells, including CCR1, CCR2, CCR5, and CXCR3, are increased within the CNS tissues of

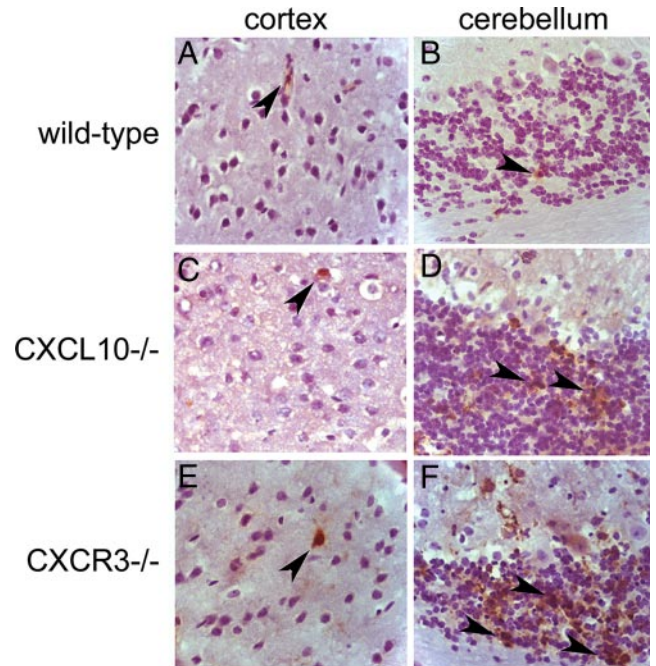


FIGURE 3. Localization of WNV Ag within CNS regions in vivo. Immunohistochemical analyses of WNV Ag (brown) within neurons (black arrowheads) of frontal cortices and cerebella from WNV-infected wild-type (A, B), CXCL10^{-/-} (C, D), and CXCR3^{-/-} (E, F) mice. All magnifications are $\times 400$. Data are representative of analyses performed on 3–4 mice per genotype.

WNV-infected mice (43, 44). Because CCR5^{-/-} also disproportionately succumb to WNV encephalitis (43), we wondered whether regional heterogeneity in the trafficking of mononuclear cells expressing specific T cell chemokine receptors or the extent of their activation might explain their nonredundant roles in the control of WNV infection within the CNS. Surface expression of CCR2, CCR5, and CD69 within CD8⁺ T cells derived from the frontal cortices and cerebella of WNV-infected mice did not reveal any differences between the two CNS regions (Fig. 4D). We also evaluated the expression of CCR1, CCR2, CCR5, and CXCR3 mRNAs within the frontal cortices and cerebella of uninfected and WNV-infected mice via quantitative RT-PCR. All chemokine receptor mRNA levels were significantly elevated over baseline levels (data not shown). In WNV-infected mice, CCR1, CCR2, and CCR5 mRNA levels did not differ significantly between the two CNS regions, whereas CXCR3 mRNA levels were significantly higher in the cerebella (Fig. 4E). Taken altogether, these data indicate that CXCR3-expressing T cells preferentially traffic into the cerebellum.

CXCL10 and CXCR3 are required for T lymphocyte recruitment into the parenchyma of the cerebellum

Given the higher levels of CXCR3 mRNA and CXCR3 receptor within tissues and on T cells derived from WNV-infected cerebellum, respectively, we directly examined the T cell trafficking patterns of CXCR3-expressing T cells in wild-type and CXCR3^{-/-} and CXCL10^{-/-} mice. A loss of CXCL10 has previously been shown to result in an overall decrease in the numbers of CD8⁺ T cells within the brains of mice with WNV encephalitis (44), although no regional analysis was performed. To more extensively evaluate the role of CXCR3 in T cell trafficking to specific brain regions, we performed flow cytometric and immunohistochemical analyses of T cell migration into the

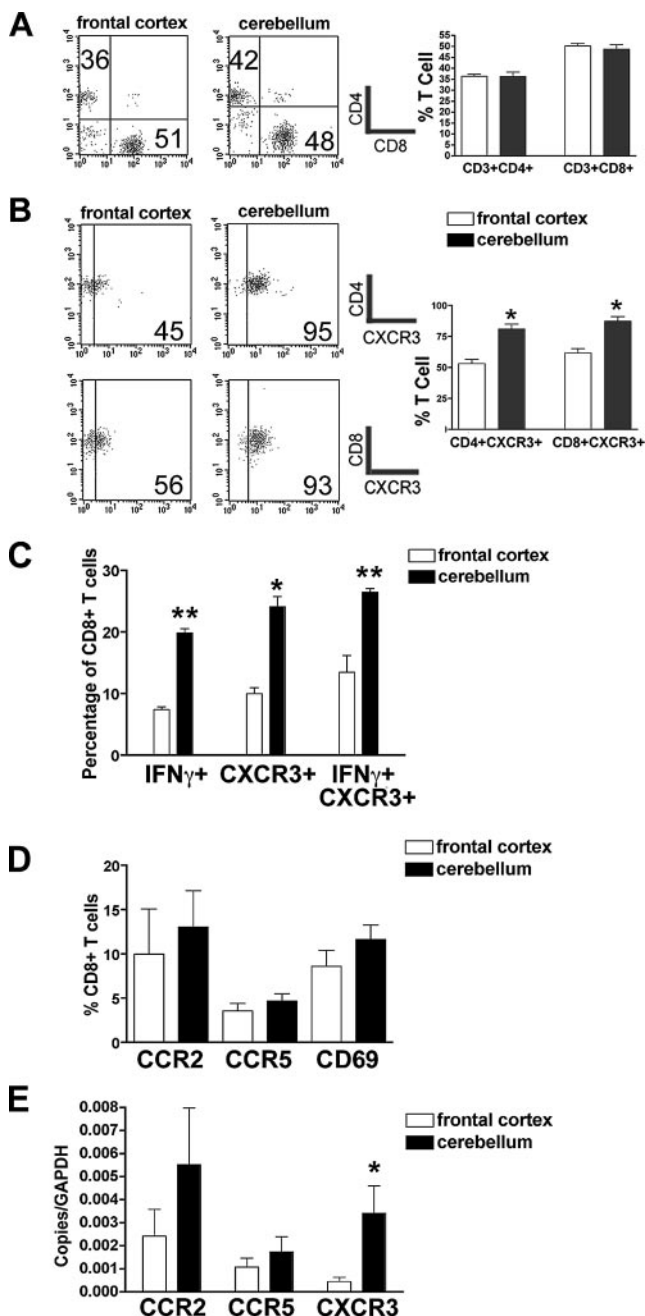


FIGURE 4. CXCR3-expressing T cells traffic into the cerebellum during WNV encephalitis. Flow cytometric analyses of percentages of CD4 and CD8 T cells (A) and CXCR3⁺CD4 and CXCR3⁺CD8 T cells (B) in the frontal cortices and cerebella of mice with WNV encephalitis. Average values of T cells are presented as percentages per five CNS tissue specimens from a representative of three experiments in which there were 6 WNV-infected mice per CNS region analyzed (\pm SEM, $*p < 0.05$). WNV-infected leukocytes derived from frontal cortices and cerebella were harvested on day 9 postinfection and stimulated ex vivo with a D^p-restricted NS4B peptide. Cells were stained with Abs against CXCR3, CD8, and IFN- γ and analyzed by flow cytometry. Percentages of IFN- γ ⁺CD8⁺, CXCR3⁺CD8⁺, and CXCR3⁺IFN- γ ⁺CD8⁺ cells from frontal cortices (white bars) and cerebella (black bars) of WNV-infected mice normalized against the percentage of CD8⁺ T cells for each brain region are shown (C). Data were obtained from two independent experiments with 3–7 mice per group. Asterisks indicate statistically significant differences between cells derived from frontal cortices and cerebella of 5-wk-old wild-type mice ($*p < 0.05$, $**p < 0.005$). Cells derived from frontal cortices (white bars) and cerebella (black bars) were also analyzed for surface expression of CCR2, CCR5, and CD69 by flow cytometry (D), and total RNA derived

frontal cortices and cerebella of wild-type and CXCR3^{-/-}-deficient mice (Fig. 5). Although the percentages of CD4⁺ T cells derived from the frontal cortices and cerebella of WNV-infected mice were not different between the two genotypes, the percentages of CD8⁺ T cells derived from frontal cortices and cerebella of WNV-infected, CXCR3-deficient mice were significantly decreased compared with those obtained from infected wild-type mice (Fig. 5A). Because we had not observed differences in viral burdens within the frontal cortices of wild-type and CXCR3^{-/-} mice, we hypothesized that the intraparenchymal trafficking of T cells within the two brain regions differed in the CXCR3^{-/-} mice. To examine this hypothesis, we examined the relative positions of CD3⁺ T cells with respect to the microvasculature via double-label immunohistochemical analyses of CNS tissues from WNV-infected wild-type, CXCR3^{-/-}, and CXCL10^{-/-} mice. In wild-type mice with WNV encephalitis, CD3⁺ T cells were observed distant from CD31-expressing endothelium within all brain regions including the frontal cortex and cerebellum (Fig. 5C). In WNV-infected CXCR3^{-/-} mice, intraparenchymal migration of CD3⁺ T cells were observed within all brain regions except the cerebellum, where CD3⁺ T cells were found only within the meninges and in association with the microvasculature (Fig. 5C). Analogous results were observed with WNV-infected CXCL10^{-/-} mice (Fig. 5C). Quantitative analyses revealed a significant decrease in the numbers of intraparenchymal CD3⁺ T cells in the cerebella of WNV-infected CXCR3^{-/-} and CXCL10^{-/-} mice compared with wild-type mice (Fig. 5B). Similar analysis of perivascular versus parenchymal T cells within the frontal cortices showed no differences among the three genotypes (data not shown). Thus, CXCR3 and CXCL10 are required for the intraparenchymal migration of T cells specifically within the cerebellum during WNV encephalitis.

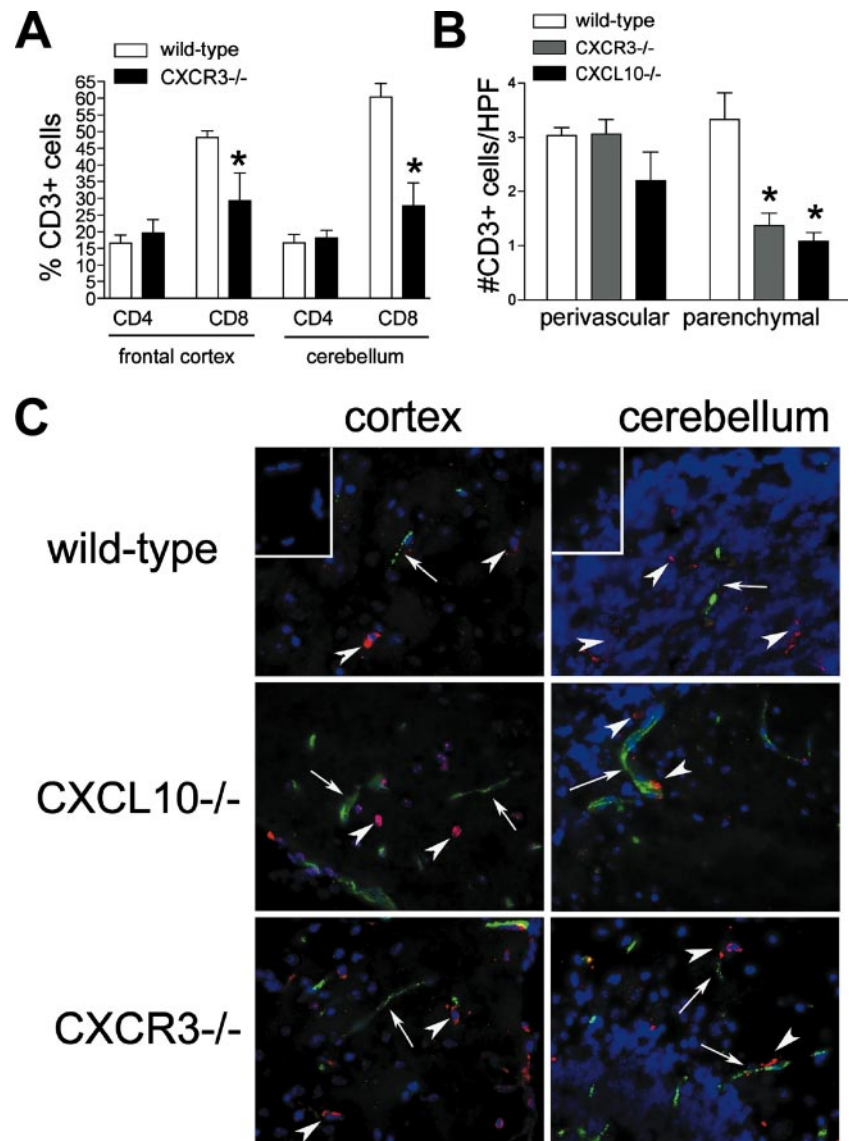
To determine whether wild-type WNV-specific CD8 T cells could rescue WNV-infected CXCR3^{-/-} mice, we performed adoptive transfer experiments. WNV-primed or naive CD8 T cells derived from wild-type mice were adoptively transferred into WNV-infected, CXCR3^{-/-} recipient mice 4 days after WNV infection, and animals were followed for survival for 30 days. Adoptive transfer of 2.5×10^6 WNV-primed CD8 T cells significantly enhanced survival of WNV-infected, CXCR3^{-/-} recipients compared with adoptive transfer of similar numbers of naive CD8 T cells (47% ($n = 19$) vs 8% ($n = 10$), respectively; $p = 0.02$). These data indicate that CD8 T cell trafficking is specifically impaired within the CNS of WNV-infected CXCR3^{-/-} mice.

CXCL10 is differentially expressed by WNV-infected, hindbrain, and forebrain neurons

In prior studies, WNV infection of the CNS was associated with the early expression of CXCL10 within Purkinje and granule cell neurons of the cerebellum (44). Given that the viral burdens between CNS compartments in WNV-infected wild-type mice do not differ significantly (Fig. 2), the increased level of CXCL10 expression within the cerebellum suggests differential responses of forebrain vs hindbrain neurons to WNV infection. To test this hypothesis directly, we evaluated neuronal expression of CXCL10 during

from frontal cortices (white bars) and cerebella (black bars) were analyzed for CCR2, CCR5, and CXCR3 mRNAs via quantitative PCR (E). Data are expressed as percentages of cells (D) and as average copies per copy of GAPDH (E) for groups of 3–6 WNV-infected mice (\pm SEM, $*p < 0.05$).

FIGURE 5. T cell trafficking is impaired within the cerebella of CXCR3- and CXCL10-deficient mice. Flow cytometric analyses of percentages of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells within the frontal cortices and cerebella of wild-type (white bars) vs CXCR3-deficient (black bars) mice (A). Average values of T cells are presented as percentages per 3–4 CNS tissue specimens from a representative of two experiments in which there were 3–4 WNV-infected mice per CNS region analyzed (\pm SEM, $*p < 0.05$). Quantitative analyses of perivascular vs parenchymal T cells within the cerebella of wild-type (white bars), CXCR3-deficient (gray bars), and CXCL10-deficient (black bars) mice are shown (B). Average values of CD3⁺ T cells were determined by analyzing the associations of CD3⁺ T cells with respect to CD31-stained vessels and counting the numbers of perivascular vs parenchymal cells per high power field (HPF). Data are expressed as average numbers of cells per HPF for 5–10 HPFs per cerebella for each of 3–4 mice per genotype (\pm SEM). C, Localization of CD3⁺ T cells (red, arrowheads) with respect to CD31⁺ vessels (green, arrows) by double-label immunohistochemistry within cortical and cerebellar tissues of WNV-infected, wild-type, CXCL10-deficient, and CXCR3-deficient mice are shown. Nuclei are counterstained with DAPI (blue). All magnifications are $\times 400$. Data are representative of analyses performed on 3–4 mice per genotype.



WNV infection in primary cortical and cerebellar neuronal cultures via quantitative RT-PCR. Examination of the level of infectious WNV produced after infection at a given multiplicity of infection did not reveal significant differences in viral yield between the two neuronal subtypes (Fig. 6A). However, WNV-infected, granule cell neurons expressed significantly higher levels of CXCL10 mRNA than did WNV-infected cortical neurons over a wide range of input virus (Fig. 6B), suggesting that the different levels of CXCL10 expression between the two neuronal subtypes do not reflect differences in infectivity. Thus, neuron-specific in-

duction of CXCL10 after WNV infection may explain the regional variations in inflammation observed during infection.

Discussion

The patterns of inflammatory infiltrates observed during viral encephalitis vary with the etiology of infection, suggesting that individual viruses may induce distinctive inflammatory responses. However, as neurons and astrocytes display regional heterogeneity in their responses and functions (55, 56), they are likely to contribute to the observed variations in leukocyte recruitment into

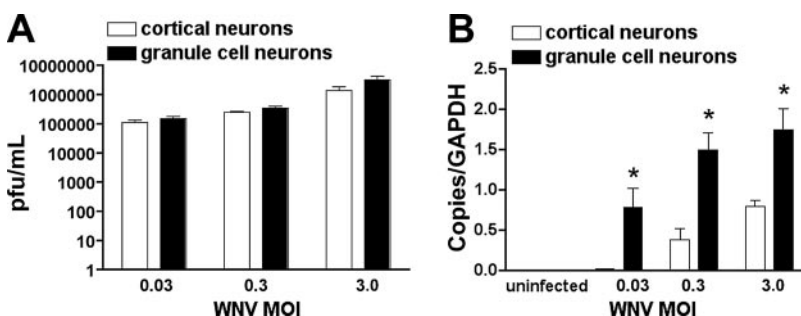


FIGURE 6. WNV induces the expression of CXCL10 in granule cell neurons. Primary cultures of cortical (white bars) and cerebellar granule cell (black bars) neurons were prepared as described in *Materials and Methods*, infected with WNV at multiplicities of infection of 0.03, 0.3, and 3, and evaluated for production of infectious WNV via plaque assay on BHK cells (A), and total RNA prepared from WNV-infected and uninfected cultures was evaluated for CXCL10 mRNA expression via quantitative RT-PCR (B). Data were derived from two separate experiments with duplicates or triplicates and expressed as mean (\pm SEM).

different CNS regions through varying capacities of chemoattractant expression. In this study, we investigated regional differences in the contribution of the chemokine receptor CXCR3 in CNS T cell trafficking in the context of WNV encephalitis. We demonstrate that WNV-mediated up-regulation of the CXCR3 ligand, CXCL10, displays differential expression depending on the type of neuron infected, and that this translates into regional differences in the trafficking of CXCR3-expressing CD8⁺ T cells and their antiviral activity. Loss of CXCR3 activation led to elevations in WNV specifically within the cerebellum, the CNS region where both CXCL10 expression and CXCR3⁺ T cell numbers were highest during WNV encephalitis. The differential expression of chemokines by virally infected neurons may therefore provide a basis for the observed nonredundant functions of chemokine receptors during WNV infection.

The chemokine receptor CXCR3, which is expressed by activated T and NK cells, binds three related, non-ELR (glutamic acid–leucine–arginine) CXC chemokines, CXCL9–CXCL11. Loss of CXCR3 activity due to targeted deletion resulted in a significant increase in mortality after WNV infection. WNV-infected, CXCL10-deficient mice display a dramatic increase in mortality without any apparent compensation by the other two ligands (44). Despite the observation that CXCL9 is essential for the recruitment of virus-specific T cells during acute CNS infection with the coronavirus, MHV (17), we observed no significant alteration in survival of WNV-infected, CXCL9-deficient mice compared with similarly infected wild-type controls. During infection with MHV, CXCL9 expression is detected in MHV-infected astrocytes and infiltrating macrophages (17). In contrast, CXCL9 was not induced in WNV-infected neurons, and peak levels coincided with the appearance of infiltrating mononuclear cells, consistent with the finding that CXCL9 is not required for the recruitment of inflammatory cells (44). Given that C57BL/6 mice do not express CXCL11 due to a frame-shift mutation (National Center for Biotechnology Information Mouse Genomic Sequence Database, accession nos. NT_109320, NW_001030791, NT_039339), our analyses would indicate that CXCL10 is the relevant CXCR3 ligand expressed in the CNS during WNV encephalitis in these animals. These results agree with recent studies examining intracranial inoculation with lymphocytic choriomeningitis virus (LCMV) or dengue virus in which both CXCL10 and CXCR3 were determined to be required for the migration of virus-specific, effector T cells into LCMV-infected meninges and into dengue-infected brains (57–59). Additionally, nonredundant roles for these two chemokines were recently demonstrated in a model of HSV-1 corneal infection in which CXCL9 alone was required for CD4 T cell infiltration into this tissue site (60).

Virologic analyses revealed significantly elevated levels of WNV in the cerebella of CXCR3-deficient mice as compared with their wild-type counterparts: no differences in viral loads were observed in the spleens, spinal cords, and frontal cortices of WNV-infected CXCR3-deficient vs wild-type mice. Consistent with this finding, immunohistochemistry revealed increased numbers of WNV-infected granule cells within the cerebella of CXCL10^{-/-} and CXCR3^{-/-} mice. We previously reported that loss of CXCL10 led to increased viral burden in both the brain and spinal cord (44). In that study, virologic analyses were not performed on different brain regions, which may explain the relatively smaller difference in net viral load observed between the wild-type and CXCL10-deficient mice (<1 log-fold) compared with the 3 log-fold increase in viral load observed in the cerebella of CXCR3-deficient mice in the present study.

Studies using various viral infectious disease models indicate that CXCR3 and its ligands are not required for the overall devel-

opment of antiviral T cell responses within secondary lymphoid organs. Normal antiviral CD8 T cell responses are generated in CXCL10^{-/-} and CXCR3^{-/-} mice infected with LCMV, influenza A, HSV-1, MHV, and murine cytomegalovirus (58–63). Similarly, no alterations were observed in the overall numbers of mononuclear cells, defects in proliferative responses and IFN- γ expression of WNV-specific CD8 cells, or in the levels of viral clearance within the spleens of WNV-infected CXCL10- and CXCR3-deficient mice as compared with similarly infected wild-type controls. Thus, it would appear that CXCR3 is dispensable for the generation of anti-viral effector CD8 T cells in the periphery during WNV infection.

In general, loss of CXCL10 or CXCR3 is associated with enhanced viral burden within infected tissues sites, but this varies with the viral etiology. Increased viral loads were observed during CNS infection of CXCL10^{-/-} and CXCR3^{-/-} mice with HSV-1, MHV, and LCMV but not during infections with influenza A or murine cytomegalovirus (26, 58, 60, 61). We observed increased viral loads specifically within the cerebella of CXCR3^{-/-}, WNV-infected mice, which correlated with the increased proportions of WNV-specific, CXCR3-expressing T cells that traffic to this site during WNV encephalitis. Most notably, loss of CXCL10 and CXCR3 was associated with a decreased parenchymal penetration of T cells within the cerebellum during WNV encephalitis. T cells in the cerebella of WNV-infected, CXCL10^{-/-}, and CXCR3^{-/-} mice remain associated with vessels and in the meninges, allowing WNV to spread more deeply into the internal granule cell layer of the cerebellum. Quantitative assessment of parenchymal vs vessel-associated T cells within the frontal cortices and cerebella of WNV-infected wild-type and CXCL10- and CXCR3-deficient mice detected significant decreases in their overall numbers of parenchymal T cells within the cerebella of the deficient mice. Interestingly, although we detected a difference in the percentages of CD4⁺ T cells expressing CXCR3 in both frontal cortices and cerebella of wild-type, WNV-infected mice, loss of CXCR3 does not affect the trafficking of CD4⁺ T cells during WNV encephalitis. This is consistent with prior studies demonstrating that CD4⁺ T cell trafficking is unimpaired in CXCR3- and CXCL10-deficient mice with experimental autoimmune encephalomyelitis, a CD4⁺ T cell-mediated autoimmune disease (64, 65). Taken together, these data indicate that CXCL10 is important for the recruitment and penetration of CXCR3-expressing CD8⁺ T cells for the control of WNV specifically within the cerebellum.

Prior studies have evaluated chemokine and cytokine mRNA levels in the cerebella and frontal cortices of WNV-infected murine brains (44). Levels and patterns of expression of CCL2, CCL5, CCL7, CXCL9, IFN- γ , and TNF- α mRNAs were similar in the two brain regions throughout the course of infection, with increased levels coinciding with the period of leukocyte entry. In contrast, CXCL10 mRNA levels were significantly higher in the cerebellum than in the frontal cortex early after WNV infection, increasing during the period of initial CNS invasion and neuronal infection. In situ analyses demonstrated that neurons are the source of CXCL10 during WNV encephalitis. Our data presented here are consistent with this in vivo data, as WNV infection of cultured granule cell neurons leads to higher levels of expression of CXCL10 than cultured cortical neurons. These data validate our in vitro system for evaluating neuronal responses to viral invasion and suggest that neurons may differentially express innate receptors that detect the presence of dsRNA during viral infections. Several cellular sensors, such as RIG-1 and MDA5, respond to viral invasion by activating preexisting transcription factors such as IFN regulatory factor 3 and the general transcription factor NF- κ B (66–68). The differential activation of these transcription

factors within virally infected neurons might underlie the observed differential patterns of cellular infiltration that occur during viral infections of the CNS. Further studies evaluating differences in innate receptor expression within various neuronal subtypes are currently in progress and should yield important insights regarding regional differences in CNS antiviral responses.

In summary, this study is the first to report that loss of a particular chemokine receptor leads to altered viral burden in a specific CNS region. Our experiments are consistent with a previous hypothesis that heterogeneity in clearance of alphavirus infection in the CNS might be due to varying abilities of neurons to respond to T cell cytokines (9). Moreover, the experiments provide a functional significance for the differences in chemokine expression and effector cell trafficking that was observed in the cerebella of animals infected with an attenuated rabies virus (27). As CXCL10 expression is variably induced by IFN- γ and TNF- α (69), and neurons may express Th1 cytokines or their receptors (70–75), the requirement of CXCR3 for effector T cell function within the cerebellum may reflect differential responses of cerebellar neurons to particular viruses. Our studies demonstrating that WNV induces higher levels of CXCL10 in granule neurons suggest that subtypes of neurons may differentially regulate the inflammatory responses that ultimately control infection. Thus, the use of pharmacologic CXCR3 antagonism, as has been proposed for the promotion of allograft survival (76, 77), may increase the risk of severe WNV cerebellar infection and lead to worse outcomes in cases of WNV encephalitis that may occur in these patients.

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Disclosures

The authors have no financial conflicts of interest.

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